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14. ABSTRACT
Eosinophilic esophagitis (EE) is an emerging worldwide food allergic disorder associated with polysensitization to multiple food allergens, resulting in greatly restricted diets and chronic gastroesophageal reflux disease-like symptoms in many individuals. EE has a strong genetic component based on the frequent presence of a familial inheritance pattern, the high sibling risk ratio (~80-fold) and the demonstration that a single nucleotide polymorphism (SNP) in the eotaxin-3 gene confers disease susceptibility. In order to dissect disease pathogenesis in humans, we are now taking genetic approaches based on genome-wide expression profile analysis of esophageal tissue, as well as a genomic analysis based on a candidate gene approach. The central hypothesis of our grant is that EE has strong genetic components that can be elucidated by a candidate gene approach focused on genes involved in asthma, allergy, and celiac disease. Using this approach, our preliminary studies have led us to hypothesize that EE susceptibility involves the IL-2/IL-21 genetic locus, a region known to be involved in immune-mediated diseases, especially autoimmunity. We have been pursuing candidate gene validation (Aim 1) and biological assessment of one lead candidate, IL-21 (Aim 2). We have identified preliminary genetic susceptibility loci involved in EE, which has led us to the identification of key potential pathogenic steps involving TSLP, IL21, and TGFb. Our results have broad implications by (1) uncovering primary molecular events involved in EE pathogenesis and, notably, how they are distinct from other forms of allergic disease such as classic food allergy (thus opening up future research directions); (2) identifying the importance of genetic components to disease susceptibility; (3) providing a framework for improved disease diagnosis (risk) and personalized predictive medicine based on genetic testing; and (4) providing therapeutic strategies based on the identified molecular pathways that may be amenable to pharmacological manipulation and/or eventual gene therapy.

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Introduction.
The central hypothesis of our grant is that eosinophilic esophagitis (EE) has strong genetic components that can be elucidated by a candidate gene approach focused on genes involved in asthma, allergy, and celiac disease. Using this approach, our preliminary studies have led us to hypothesize that EE susceptibility involves the IL-2/IL-21 genetic locus, a region known to be involved in immune-mediated diseases, especially autoimmunity. We have been pursuing candidate gene validation (Aim 1) and biological assessment of one lead candidate, IL-21 (Aim 2).

Body.
Aim 1. Validate the Associations Between EE and Asthma/Allergy/Celiac Candidate Genes

- Our prior work on the TGFB1 and TGFBR SNPs was not validated in replication cohorts.
- We are validating the role of IL-2/IL-21 genes, as well as other genes involved in regulating immune responses, such as the TGFB1 receptor II, in susceptibility to EE.

Please see the Tables below for specific data:

Table 2. SNPs in the IL21 locus associate with EoE

CCHMC EE (n=226) vs. CHOP controls (n=316)									
SNP	**CHR**	**BP**	**Alleles**	**Gene**	**Location**	**Left gene**	**Right gene**	***P*-values**	**OR**
rs12642902	4	123727951	A/G	NA	NA	IL2	IL21	0.02	1.27
rs6835745	4	123730462	G/T	NA	NA	IL2	IL21	0.04	1.25
rs975405	4	123740630	C/T	IL21	intron	IL2	LOC729338	0.04	1.23

- We are examining candidate genes using a broad spectrum approach by employment of a custom Illumina ImmunoChip, that contains ~200,000 SNPs that are focused on regions that have previously achieved genome-wide significance following GWAS for a series of autoimmune diseases. This analysis is fairly mature and a near complete data set of the major identified locus, using a discovery and replication cohort of patients and controls is provided in Table 2.

Table 2. Common variants associated with pediatric Eosinophilic Esophagitis.

			Discovery				Replication					
			MAF				MAF					
dbSNP	Chr BP	ncbi Gene	Cases	Controls	OR	P value *	Cases	Controls	OR	P value *	Fisher's combined	
rs4130548	Chr 1 78463868	LOC100 288957	0.287	0.397	0.61	4.80E-05	0.368	0.344	0.97	0.83		
rs12143327	Chr 1 112610895		0.143	0.077	1.95	8.60E-05	0.089	0.089	1.02	0.92		
rs10926865	Chr 1 241032699		0.5	0.419	1.82	9.20E-05	0.509	0.412	2.17	1.40E-05	2.71E-08	1q44
1kg_1_241047614	Chr 1 241047614		0.384	0.248	3.14	9.80E-12	0.359	0.23	2.8	3.20E-08	5.38E-18	1q44
rs12404844	Chr 1 241049304		0.502	0.417	1.9	3.20E-05	0.512	0.426	2.02	8.50E-05	5.71E-08	1q44
rs2214889	Chr2 101929203	MAP4K4 /IL1R2	0.441	0.332	1.6	2.80E-05	0.368	0.357	0.95	0.67		
rs1010329	Chr 2 101931502	MAP4K4 /IL1R2	0.439	0.332	1.59	3.90E-05	0.368	0.354	0.95	0.72		
rs10181476	Chr 2 101932700	MAP4K4 /IL1R2	0.439	0.332	1.59	3.90E-05	0.367	0.345	1	0.98		
rs4851513	Chr 2 101949567	MAP4K4 /IL1R2	0.237	0.337	0.62	1.00E-04	0.249	0.271	0.93	0.6		
rs9860962	Chr 3 51294429	DOCK3	0.055	0.12	0.43	1.40E-07	0.044	0.0844	0.5	0.012	3.56E-08	3p21
NA	Chr 3 189580797	LPP	0.011	0.066	0.15	6.10E-05	0.084	0.06	1.4	0.15		
rs9268861	Chr 6 32429894	HLA-DRA	0.126	0.22	0.49	1.10E-05	0.196	0.228	0.71	0.032	5.77E-06	6p21.3
rs1559797	Chr8 74870647	TCEB1	0.249	0.361	0.59	2.10E-05	0.311	0.335	0.9	0.47		
rs4738402	Chr 8 74880319	TCEB1	0.249	0.361	0.59	2.10E-05	0.313	0.337	0.9	0.42		
rs8021089	Chr 14 78171544	NRXN3	0.106	0.048	2.34	3.00E-05	0.077	0.071	1.46	0.13		
rs6507	Chr 19 483066	CDC34	0.114	0.042	2.89	4.40E-07	0.074	0.094	0.76	0.26		

bSNP, Build 130 rs number; CHR - BR, chromosomal location National Center for Biotechnology Information (NCBI) Build 36; MAF, minor allele frequencies; P values, P value of logistic regression under an additive model adjusted for 6 principle components; OR, odds ratio.

The association with the 4q27 locus was validated. Currently, experiments are carried in control cohorts with various disease states including atopy and asthma to control for various background effects. In terms of using the Asthma Chip for a similar analysis, the ImmunoChip provides a more robust selection of genes and SNPs (for example ~200,000 SNPs vs ~1000 SNPs on the Asthma chip). Nevertheless, we now have a second generation Asthma Chip, which has now been generated and delivered to us and we are in the process of screening EoE and control cohorts.

Aim 2. Elucidate the Role of IL-21 in EE.

- Levels of IL-21 are shown in Figure 1 and indicate that IL-21 and IL-21R expression within the esophagus are unchanged in active EoE patients compared to normal (NL) controls (Figure 1). However, these data do not address the potential for IL-21 or IL-21R expression to be altered in other tissues (blood, skin, etc.) that could contribute to esophageal inflammation in EoE. Therefore, we are currently investigating IL-21 and IL-21R genes and protein expression in the blood of EoE patients and NL controls by ELISA, quantitative PCR, and flow cytometry.

- We have examined EE induction in mice following intranasal challenge with *Aspergillus fumigatus* extract and found that IL-21 regulates the kinetics of allergen-induced pulmonary inflammatory responses (Figure 2). Reduced total cells and total eosinophils in the bronchoalveolar lavage fluid (BALF) were observed in *Il21r*-/- mice compared to wild-type (WT) mice only at early times (six vs. nine challenges) in the experimental EoE model. Moreover, esophageal eosinophilia was reduced in IL-21R-/- mice following allergen challenge (Figure 3). We hypothesized that the reduced esophageal eosinophilia in the *Il21r*-/- mice was associated with reduced levels of eosinophil-recruiting chemokines. Indeed, we observed decreased esophageal expression of the key eosinophil chemoattractant eotaxin-1 in the *Il21r*-/- mice compared to wild-type mice following allergen challenge. These data support our hypothesis that IL-21 plays an upstream role in mediating the esophageal inflammation associated with EoE. To further address this hypothesis, we examined the production of the Th2 cytokine interleukin-4 (IL-4) following *A. fumigatus* restimulation of cells isolated from lung draining lymph nodes from WT and *Il21r*-/- mice following intranasal challenge. It is postulated that the migration of Th2 cells from the inflamed lung into the lung draining lymph nodes is a key step in the development of secondary tissue (i.e., esophageal) eosinophilia. Here, we demonstrate that restimulation with *A. fumigatus* results in significantly greater levels of IL-4 production in allergen (but not saline) challenged WT mice compared to allergen challenged *Il21r*-/- mice (Figure 4).

- The presence of specific autoantibodies in EE sera has been measured by examining for the presence of IgE, IgG, and IgM that shows specific binding pattern to lysates from esophageal epithelial cells, as well as esophageal tissue from patients with active EE. This analysis has not shown a reproducible titre of auto-antibodies (Figure 4).

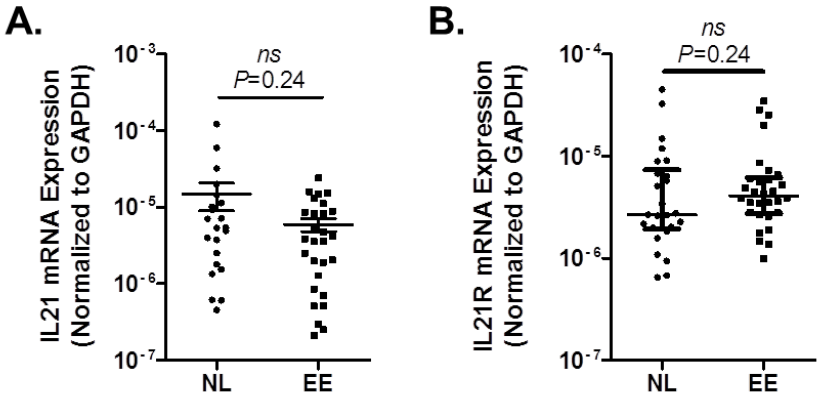

Figure 1. IL-21 and IL-21R expression are unchanged in EoE. Quantitative PCR analysis of IL-21 (A) and IL-21R (B) in esophageal biopsies from normal (NL) controls and active EoE patients.

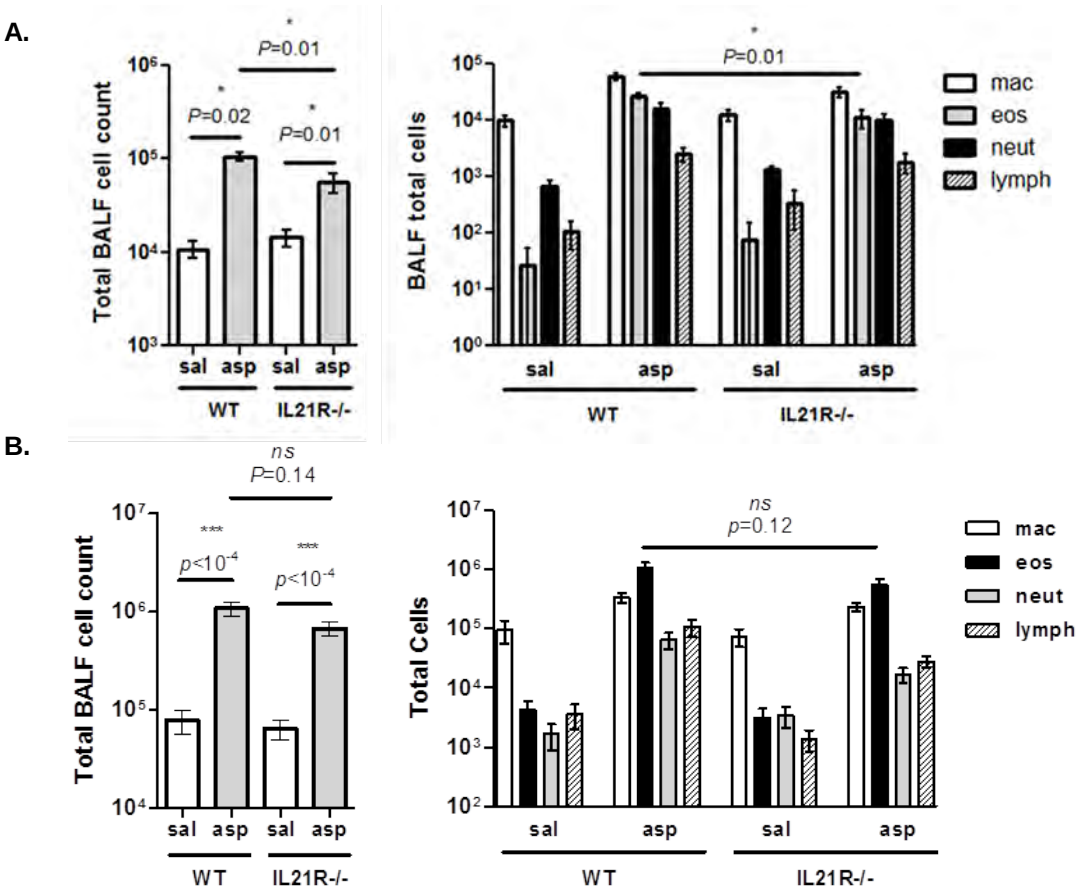

Figure 2. IL-21R-/- mice have delayed pulmonary inflammation in response to *A. fumigatus* allergen. Lung inflammation following six (A) or nine (B) allergen challenges, as assessed by cellular content (total cells, left panel, and differential cell counts, right panel) in bronchoalveolar lavage fluid (BALF) in IL-21R-/- mice compared to wild-type (WT).

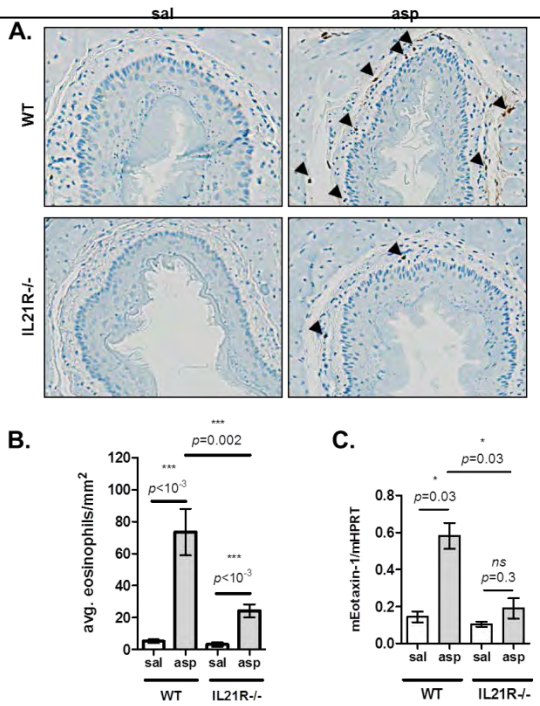

Figure 3. IL-21R-/- mice have reduced esophageal eosinophilia following intranasal allergen-challenge. Wild-type (WT) or IL21R-deficeint mice were sacrificed following nine intranasal challenges with saline or *A. fumigatus* extract. Immunohistochemical staining for major basic protein in esophageal tissue (arrowheads) in WT than IL21R-/- mice (A) is quantified morphometrically in (B). Quantitative PCR (C) for esophageal expression of eotaxin-1 in IL21R-/-and WT mice following allergen challenge.

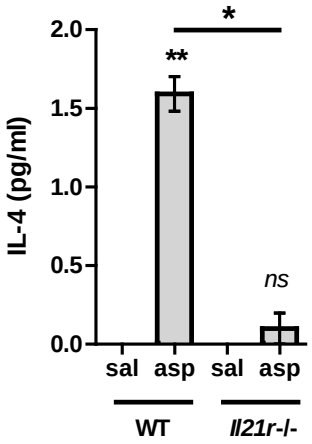

Figure 4. *A. fumigatus* restimulation induces IL-4 production from lymph node cells in allergen-challenged WT but not *Il21r-/-* mice. Lymph node cells from saline or allergen challenged WT or Il21r-/- mice were treated for 24 hours with 100 ng/mL *A. fumigatus* extract and IL-4 production was measured in the supernatant by ELISA. *, $p < 0.05$; ns (not significant) and ** ($p < 0.005$) compared to saline challenged mice (three mice per group).

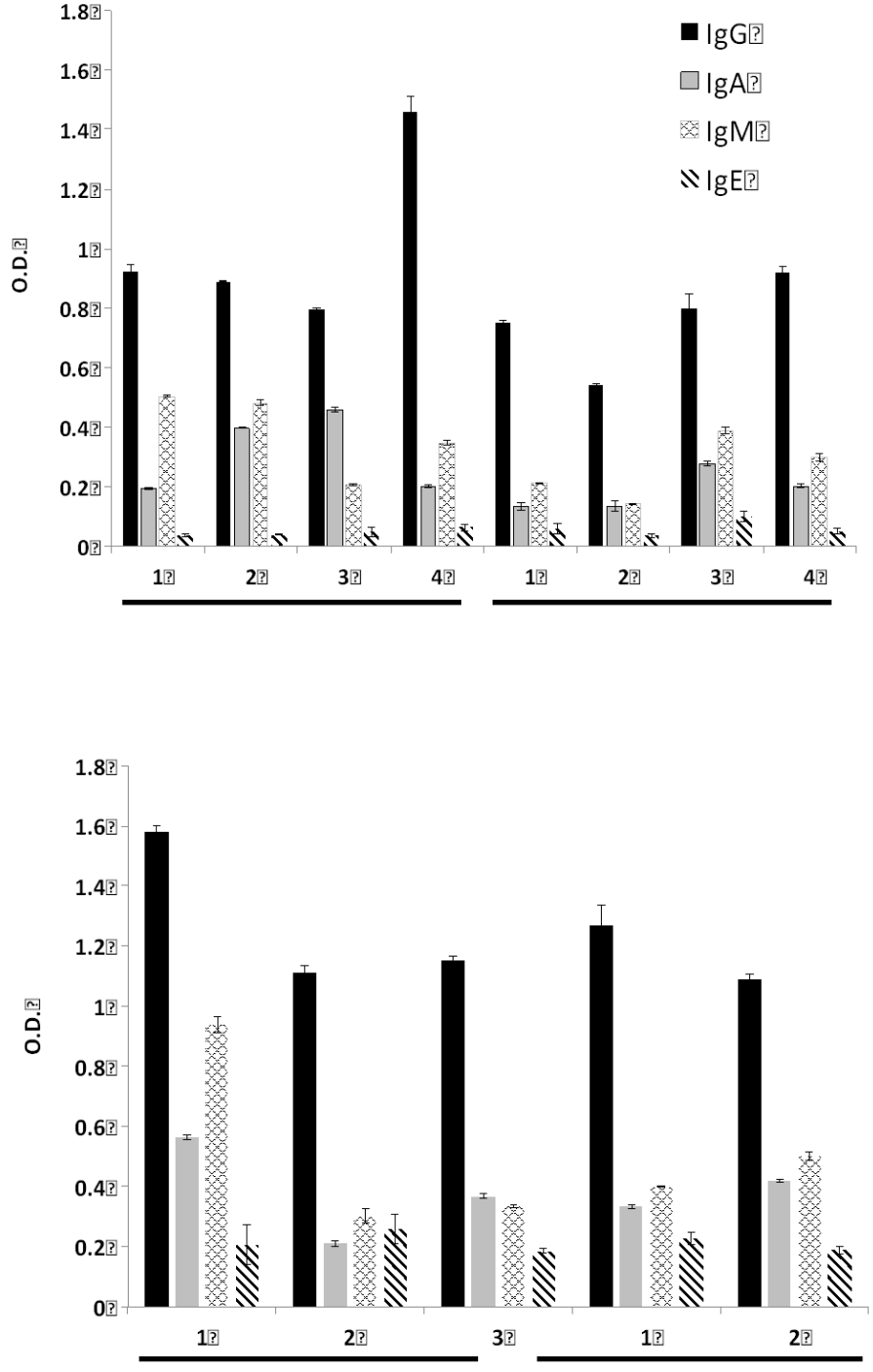

Figure 4. Autoantibody detection in eosinophilic esophagitis patient sera. A, Esophageal cell line lysate was plated on an ELISA plate. Patient or control sera was applied and followed with respective anti-Ig secondary antibody. **B,** Patient or control biopsy lysate was plated on an ELISA plate. Matched sera was applied and followed with respective anti-Ig secondary antibody. Negative control (without lysate) was subtracted from the data.

Key Research Accomplishments.

- EE Case Control Analysis vs. Distinct Control Groups identifies SNPs in TSLP and a variety of other candidate genes.
- IL-21R deficient mice are demonstrated to have protection from allergen-induced EE.
- Genome-wide significance of X and Y gene locus with EoE susceptibility.

Reportable Outcomes.

1) Sherrill JD, Gao PS, Stucke EM, Blanchard C, Collins MH, Putnam PE, Franciosi JP, Kuchner JP, Abonia, JP, Assa'ad AH, Kovacic MB, Biagini Myers JM, Bochner BS, He H, Hershey GK, Martin LJ, Rothenberg ME. Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis. *J Allergy Clin Immunol*. 2010;126(1):160-5.e3. PMCID: PMC2904342.
2) Sherrill JD, Rothenberg ME. Genetic dissection of eosinophilic esophagitis provides insight into disease pathogenesis and treatment strategies. *J Allergy Clin Immunol*. 2011;128(1):23-32. PMCID: PMC3129465.
   Please note: The Department of Defense was cited in the Reportable Outcomes Reference items #1 and #2 as (1) a portion of funds were used for recruitment of DNA that was analyzed; (2) cited Reference #1 uses a DNA chip that contains 738 SNPs within immune-based genes that have impact on the proposed study; and (3) Aim 1 proposed to use a broad based custom chip approach and this was preliminarily done in the Sherrill thymic stromal lymphopoietin *J Allergy Clin Immunol,* Reference #2.
3) Sherrill J, Martin L, Blanchard C, Annaiah K, Spergel; J, Hakonarson H, Rothenberg M. Genetic risk variants for celiac disease in the IL2/IL21 locus are associated with eosinophilic esophagitis. *J Allergy Clin Immunol*. Feb2010;125(2)Supplement 1:AB160. Abstract. http://www.jacionline.org/article/S0091-6749(09)02424-5/fulltext
   Please note: Data presented at AAAAI Annual Meeting on March 1, 2011.
4) Tissue Repository - we are collecting DNA from EE patients and can estimate 150 EE DNA samples.

Conclusion.

There is currently a paucity of molecular and genetic insight into EE, and there is currently no approved drug for this food allergy-associated disease, highlighting the need for innovative fundamental studies focused on EE. We have identified preliminary genetic susceptibility loci involved in EE, which has led us to the identification of key potential pathogenic steps involving TSLP, IL-21, and TGF-beta. Our results have broad implications by (1) uncovering primary molecular events involved in EE pathogenesis; notably, how they are distinct from other forms of allergic disease, such as classic food allergy (thus, opening up future research directions); (2) identifying the importance of genetic components to disease susceptibility; (3) providing a framework for improved disease diagnosis (risk) and personalized predictive medicine based on genetic testing; and (4) providing therapeutic strategies based on the identified molecular pathways that may be amenable to pharmacological manipulation and/or eventual gene therapy.

References.

1. Sherrill JD, Gao PS, Stucke EM, Blanchard C, Collins MH, Putnam PE, Franciosi JP, Kuchner JP, Abonia, JP, Assa'ad AH, Kovacic MB, Biagini Myers JM, Bochner BS, He H, Hershey GK, Martin LJ, Rothenberg ME. Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis. *J Allergy Clin Immunol*. 2010;126(1):160-5.e3. PMCID: PMC2904342.

2. Sherrill JD, Rothenberg ME. Genetic dissection of eosinophilic esophagitis provides insight not disease pathogenesis and treatment strategies. *J Allergy Clin Immunol*. 2011;128(1):23-32. PMCID: PMC3129465.
3. Sherrill J, Martin L, Blanchard C, Annaiah K, Spergel; J, Hakonarson H, Rothenberg M. Genetic risk variants for celiac disease in the IL2/IL21 locus are associated with eosinophilic esophagitis. *J Allergy Clin Immunol*. Feb2010;125(2)Supplement 1:AB160. Abstract. http://www.jacionline.org/article/S0091-6749(09)02424-5/fulltext
Please note: Data presented at AAAAI Annual Meeting on March 1, 2011.

Appendices.

Appendix 1. List of personnel (not salaries) receiving pay from the research effort.

Appendix 2. Sherrill JD, Gao PS, Stucke EM, Blanchard C, Collins MH, Putnam PE, Franciosi JP, Kuchner JP, Abonia, JP, Assa'ad AH, Kovacic MB, Biagini Myers JM, Bochner BS, He H, Hershey GK, Martin LJ, Rothenberg ME. Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis. *J Allergy Clin Immunol*. 2010;126(1):160-5.e3. PMCID: PMC2904342.

Appendix 3. Sherrill JD, Rothenberg ME. Genetic dissection of eosinophilic esophagitis provides insight not disease pathogenesis and treatment strategies. *J Allergy Clin Immunol*. 2011;128(1):23-32. PMCID: PMC3129465.

Appendix 4. Sherrill J, Martin L, Blanchard C, Annaiah K, Spergel; J, Hakonarson H, Rothenberg M. Genetic risk variants for celiac disease in the IL2/IL21 locus are associated with eosinophilic esophagitis. *J Allergy Clin Immunol*. Feb2010;125(2)Supplement 1:AB160. Abstract. http://www.jacionline.org/article/S0091-6749(09)02424-5/fulltext
Please note: Data presented at AAAAI Annual Meeting on March 1, 2011.

Appendix 1. List of personnel (not salaries) receiving pay from the research effort.

Employee Name		Job Description
Besse, John A.		RESEARCH ASST III
Bouffi PHD, Carine		RESEARCH FELLOW
Hershey MD-PHD, Gurjit Khurana		PROFESSOR - FACULTY
Itskovich PHD, Svetlana S.		RESEARCH FELLOW
Martin PHD, Lisa		ASSOCIATE PROFESSOR
Rothenberg MD-PHD, Marc E		PROFESSOR-DIV DIR
Wen PHD, Ting		RESEARCH FELLOW
Zimmermann MD, Nives		ASSOCIATE PROFESSOR

Appendix 2.

Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis

Joseph D. Sherrill, PhD,[a] Pei-Song Gao, MD, PhD,[b] Emily M. Stucke, BA,[a] Carine Blanchard, PhD,[a] Margaret H. Collins, MD,[c] Phil E. Putnam, MD,[d] James P. Franciosi, MD,[d] Jonathan P. Kushner, MD,[e] J. Pablo Abonia, MD,[a] Amal H. Assa'ad, MD,[a] Melinda Butsch Kovacic, PhD,[f] Jocelyn M. Biagini Myers, PhD,[f] Bruce S. Bochner, MD,[b] Hua He, MS,[g] Gurjit Khurana Hershey, MD, PhD,[a,f] Lisa J. Martin, PhD,[g,h] and Marc E. Rothenberg, MD, PhD[a] *Cincinnati, Ohio, and Baltimore, Md*

Background: The genetic cause of eosinophilic esophagitis (EE) has been largely unexplored until a recent genome-wide association study identified a disease susceptibility locus on 5q22, a region that harbors the thymic stromal lymphopoietin *(TSLP)* gene. However, it is unclear whether the observed genetic associations with EE are disease-specific or confounded by the high rate of allergy in patients with EE. In addition, the genetic contributions of other allergy-associated genes to EE risk have not been explored.

Objective: We aimed to delineate single nucleotide polymorphisms (SNPs) that associated with EE apart from allergy.

Methods: We used a custom array containing 738 SNPs in 53 genes implicated in allergic responses, immune responses, or both to genotype 220 allergic or 246 nonallergic control subjects and a discovery cohort of 170 patients with EE. We replicated a statistically significant SNP association in an independent case-control cohort and examined the induction of the candidate gene in primary esophageal epithelial cells.

Results: A single SNP residing in the *TSLP* gene reached Bonferroni linkage disequilibrium–adjusted significance but only when patients with EE were compared with allergic control subjects (rs10062929; $P = 4.11 \times 10^{-5}$; odds ratio, 0.35). A nonsynonymous polymorphism in the thymic stromal lymphopoietin receptor *(TSLPR)* gene on Xp22.3 and Yp11.3 was significantly associated with disease only in male patients with EE. Primary esophageal epithelial cells expressed *TSLP* mRNA after Toll-like receptor 3 stimulation.

Conclusion: These data collectively identify *TSLP* as a candidate gene critically involved in EE susceptibility beyond its role in promoting T_H2 responses. (J Allergy Clin Immunol 2010;126:160-5.)

Key words: *Eosinophilic esophagitis, thymic stromal lymphopoietin, single nucleotide polymorphism, allergy, cytokine receptor–like factor 2, Toll-like receptor 3*

Eosinophilic esophagitis (EE) is a chronic T_H2-associated inflammatory disease of the esophagus that affects at least 4 in 10,000 persons.[1] Although symptomatically resembling gastroesophageal reflux disease, EE is clinically defined as esophageal eosinophilia (≥15 intraepithelial eosinophils per high-powered field) in the absence of abnormal acid reflux disease (assessed by normal pH monitoring of the distal esophagus or persistent esophagitis during high-dose acid suppression therapy).[2] Another distinguishing feature is the high rate of atopic diseases, including asthma, eczema, and allergic rhinitis, and sensitivities to environmental and food allergens within both pediatric and adult EE populations.[3,4] Consistent with an immune-based mechanism of disease induction, the most effective therapies used to manage EE are food antigen avoidance and swallowed glucocorticoid treatment. However, some patients with EE are refractory to glucocorticoid treatment,[5] suggesting an inherent resistance with a potential genetic basis.

Reports of EE and esophageal dilatation in relatives of patients with EE suggest that the incidence of EE and associated esophageal dysfunction is common among related subjects.[1,6] Molecular analysis of the genetics behind EE was initiated in a study by Blanchard et al,[7] which identified a characteristic gene expression profile within the esophagi of patients with EE, termed the EE transcriptome, that was distinct from that observed in other forms of chronic esophagitis. Interestingly, the EE transcriptome was consistent across sex, age, and familial or nonfamilial inheritance patterns and was independent of atopic status, suggesting a

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Supported in part by National Institutes of Health grant U19 AI070235, National Institutes of Health grant R01 DK076893, Public Health Service grant P30 DK0789392, the Department of Defense, the Food Allergy Project, the Buckeye Foundation, and the Campaign Urging Research for Eosinophilic Disorders (CURED) Foundation. J.D.S. is supported by a T32 National Institutes of Health training grant (HL091805). P.G., B.S.B., and M.E.R. were supported by a Dana Foundation Human Immunology Consortium Grant.

Disclosure of potential conflict of interest: M. H. Collins has consultant and reviewer arrangements with GlaxoSmithKline, Ception Therapeutics, and Meritage Pharma. J. P. Abonia receives research support from the National Institutes of Health, Ception Therapeutics, and the Children's Digestive Health and Nutrition Foundation. A. H. Assa'ad receives research support from GlaxoSmithKline and is a volunteer member of the medical board of directors for the American Partnership for Eosinophilic Disorders. L. J. Martin receives research support from the National Institutes of Health. M. E. Rothenberg is on the speakers' bureau for Merck; has consultant arrangements with Merck, Centocor, Ception Therapeutics, Nycomed, Array Biopharma, Biocrystal Pharmaceuticals, Endo Pharmaceuticals, and Pieres AG; receives research support from the National Institutes of Health, the Food Allergy and Anaphylaxis Network, and the Dana Foundation; is on the Medical Advisory Board for American Partnership for Eosinophilic Disorders; and is on the Executive Council for the International Eosinophil Society. The rest of the authors have declared that they have no conflict of interest.

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0091-6749/$36.00

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Abbreviations used
CCED: Cincinnati Center for Eosinophilic Disorders
CCHMC: Cincinnati Children's Hospital Medical Center
EE: Eosinophilic esophagitis
GWAS: Genome-wide association study
LD: linkage disequilibrium
MAF: Minor allele frequency
OR: Odds ratio
SNP: Single nucleotide polymorphism
TLR3: Toll-like receptor 3
TSLP: Thymic stromal lymphopoietin
TSLPR: Thymic stromal lymphopoietin receptor

common disease mechanism despite phenotypic variations.[6,7] The most highly expressed gene in the EE transcriptome is eotaxin-3 (53-fold increase), an eosinophil and mast cell chemoattractant; indeed, eotaxin-3 levels correlate with both esophageal eosinophil and mast cell levels.[7] Furthermore, evidence is mounting that disease pathogenesis is mediated by the interaction of T_H2 cells and esophageal epithelial cells because the T_H2 cytokine IL-13 induces a large fraction of the EE transcriptome (including eotaxin-3) in esophageal epithelial cells.[8,9] A recent genome-wide association study (GWAS) identified 5q22 as a susceptibility locus for pediatric EE.[10] Notably, although 2 genes (thymic stromal lymphopoietin *[TSLP]* and *WDR36*) were present in one haplotype block that associated with EE, evidence was provided that *TSLP* was the stronger candidate gene because of its overexpression in the esophagi of patients with EE and its known biological activity as a key regulator of allergic sensitization.[10] Yet *TSLP* has previously been implicated in various atopic responses,[11-13] and thus it is not clear whether the EE association with *TSLP* is reflective of a general association with atopic processes or whether it is specific to EE.

In the present study we sought to identify genetic variants that associated with EE using a broad-spectrum candidate gene approach involving a panel of 738 single nucleotide polymorphisms (SNPs) in allergy-associated molecules, especially epithelial gene products, including TSLP. Furthermore, we aimed to identify genetic variants that associated with EE independent of allergy. To address this question, we genetically compared patients with EE with a set of clinically defined allergic and nonallergic control groups. Compared with all genetic variants tested, we demonstrate that genetic variants within *TSLP* are associated with EE independent of allergy. Additionally, we present further evidence for the importance of *TSLP* in patients with EE by demonstrating that a genetic variant in the thymic stromal lymphopoietin receptor *(TSLPR)* also contributes to EE susceptibility. Furthermore, we show that primary esophageal epithelial cells express *TSLP* mRNA in response to Toll-like receptor 3 (TLR3) activation.

METHODS

Study participants

The discovery EE case cohort consisted of 172 patients who were recruited by the Cincinnati Center for Eosinophilic Disorders (CCED). DNA samples were collected at the time of endoscopy or at follow-up from blood or saliva specimens. Patients were identified with clinically diagnosed EE (≥24 eosinophils per high-power field). All patients with EE self-reported race as white. The male/female ratio was 2.18, and the mean age at diagnosis was 9.39 years (SD, 8.60 years). Approximately 85% of the discovery cohort was composed of patients characterized in a recent EE GWAS.[10] The percentages of patients with diagnosed asthma, allergic rhinitis, or eczema were 31%, 53%, and 39%, respectively. Approximately 50% of these patients with EE were receiving acid-suppressive therapy, and 18% were taking swallowed glucocorticoid treatment at the time of endoscopy. The discovery control cohorts were recruited through the Greater Cincinnati Pediatric Clinic Repository and Genomic Control Cohort at Cincinnati Children's Hospital Medical Center (CCHMC). All consenting patients visiting various clinics at CCHMC, including the allergy, immunology, and pulmonary clinics, provided buccal swabs or saliva samples for DNA isolation. Information regarding patient history was collected through questionnaires pertaining to allergy symptoms. All control subjects had self-reported race as white. The allergic control cohort ($n = 227$) was defined as nonasthmatic subjects having clinically diagnosed atopic rhinitis or atopic dermatitis or a self-reported history of environmental allergies, hay fever, or eczema. The nonallergic control group ($n = 246$) had no personal or family history of asthma or a personal history of environmental allergies, allergic rhinitis, or atopic dermatitis.

The replication EE case cohort consisted of 122 independent patients with similar characteristics as the EE discovery cohort. This cohort was also collected at the CCED by using the procedures described above. All patients with EE in the replication cohort self-reported race as white, and 72% of these patients were included in the EE GWAS. The replication control cohort consisted of 119 subjects. This cohort was comprised of consenting CCHMC employees who reported race as white with no self-reported personal history of gastroesophageal reflux disease or other gastrointestinal symptoms. The percentage of subjects within this group with self-reported allergy was approximately 44%. These studies were approved by the CCHMC Institutional Review Board.

Candidate gene and SNP selection

For the discovery phase, we used a custom Illumina SNP chip ($n_{SNP} = 768$) designed to interrogate genes with a role in allergy-related signaling pathways, epithelial cell function, or both based on current literature searches as part of National Institutes of Health grant U19 AI070235. The *TSLP* gene was submitted for inclusion in the initial chip design by Dr Yong-Jun Liu (University of Texas, M.D. Anderson Cancer Center, Houston, Tex); a total of 9 *TSLP* SNPs were included on the chip. In addition to candidate gene SNPs, 30 ancestry informative markers were also included. Genotyping with the Illumina GoldenGate Assay (http://www.illumina.com) system was performed at the CCHMC Genetic Variation and Gene Discovery Core. Genotypes were assigned by using Illumina's BeadStudio 2 Software (San Diego, Calif). SNPs that exhibited differences in minor allele frequencies (MAFs > 0.1) between plates were manually examined to ensure consistent clustering patterns. For the replication set, *TSLP* SNPs were genotyped with the ABI TaqMan allelic discrimination assays (Applied Biosystems, Foster City, Calif). The initial *TSLPR* sequencing was performed with the ABI 3700 DNA Analyzer. *TSLPR* SNPs were genotyped with the ABI TaqMan allelic discrimination assays (Applied Biosystems) in the replication case-control cohorts, as well as in additional DNA samples from patients with EE and healthy control subjects collected through the CCED.

Statistical analyses

All analyses were performed separately among the allergic and nonallergic control cohorts. No *TSLP* SNPs failed Hardy-Weinberg equilibrium in the control dataset ($P < 10^{-3}$), were removed for poor genotype calling (missing rate >10%), or were excluded with MAFs of less than 10%. A single *TSLP* SNP was removed before analysis because of poor clustering. To adjust for multiple testing, we applied a Bonferroni adjustment after correcting for linkage disequilibrium (LD) correlation among the SNPs that passed quality control. This approach is less conservative than the standard Bonferroni adjustment because it incorporates an LD block–based correction to reduce the total number of independent tests, thus preventing type I error inflation.[14] Filtering of subjects with greater than 20% missing genotypic information over the chip resulted in the removal of 2 patients with EE and 7 allergic control subjects before analysis, yielding a total of 170 cases and 220 allergic and 246 nonallergic control

TABLE I. *TSLP* SNPs in patients with EE compared with allergic, nonallergic, or both control subjects

		Patients with EE (n = 170)	Allergic control subjects (n = 220)			Nonallergic control subjects (n = 246)			Allergic and nonallergic control subjects (n = 466)		
SNP*	BP†	MAF‡ cases	MAF control subjects	OR§	*P* value	MAF control subjects	OR	*P* value	MAF control subjects	OR	*P* value
rs3806932	110433574	0.33	0.40	0.72	3.27E-02	0.41	0.70	1.55E-02	0.41	0.70	9.02E-03
rs3806933	110434641	0.31	0.41	0.63	3.29E-03	0.40	0.70	1.56E-02	0.40	0.66	2.38E-03
rs2289276	110435406	0.24	0.25	0.94	7.40E-01	0.27	0.83	2.69E-01	0.26	0.86	3.43E-01
rs1898671	110435901	0.45	0.33	1.61	1.26E-03	0.35	1.48	7.35E-03	0.34	1.57	5.52E-04
rs10062929	110436078	0.08	0.17	0.35	4.11E-05	0.13	0.54	1.27E-02	0.15	0.45	5.79E-04
rs2289277	110436966	0.31	0.42	0.61	1.90E-03	0.40	0.69	1.05E-02	0.41	0.64	1.35E-03
rs11466749	110440484	0.11	0.19	0.48	9.29E-04	0.15	0.71	1.12E-01	0.17	0.59	8.47E-03
rs11466750	110440793	0.09	0.18	0.43	3.37E-04	0.13	0.63	5.25E-02	0.15	0.52	3.08E-03

Presented are the associated *P* values for *TSLP* SNPs between patients with EE versus 3 control groups (allergic, nonallergic, or combined).
*dbSNP Build 130 rs number.
†*BP*, Base pair; chromosomal location National Center for Biotechnology Information (NCBI) Build 36.
‡MAF in patients with EE or the specified control group.
§OR for the specified control group compared with patients with EE.

subjects that entered the analyses. Principal component analysis was performed with 30 ancestry informative markers and the EIGENSTRAT software (http://genepath.med.harvard.edu/~reich/EIGENSTRAT.htm) to account for potential population stratification/confounding or admixture in these samples. The principal component score for each subject was included as a covariate in all models along with age and sex in logistic regression models. As a general association screen, we tested for the additive models of single-SNP analysis, which assumes that each copy of the risk allele will increase disease prevalence. Unconditional logistic regression was used to calculate *P* values and odds ratios (ORs) for each SNP by using the software PLINK (version 1.05).[15] Statistical analyses of the *TSLPR* SNPs (dominant model) and the *TSLP* SNPs (Cochran-Armitage trend test) in the replication cohorts were also performed in PLINK. The Fisher method,[16] which calculates the combined probability from independent tests addressing the same hypothesis, was used in the meta-analysis of the discovery and replication cohorts.

TSLP expression in stimulated esophageal epithelial cells

Primary esophageal epithelial cells were cultured from patients' esophageal biopsy specimens, as previously described.[9] Cells were grown in hydrocortisone-free media 24 to 48 hours before stimulation. Cells were then treated with 10 or 100 μg/mL poly I:C (InvivoGen, San Diego, Calif) for 3 or 8 hours. RNA was isolated with TRIzol (Invitrogen, Carlsbad, Calif), and cDNA was synthesized with iScript (Bio-Rad Laboratories, Hercules, Calif). RT-PCR analysis for *TSLP* expression was performed with the following primers: forward, 5′-cccaggctattcggaaactca-3′; reverse, 5′-acgccacaa tccttgtaattgtg-3′. Graphed data were normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* expression from 3 to 4 independent experiments performed in duplicate.

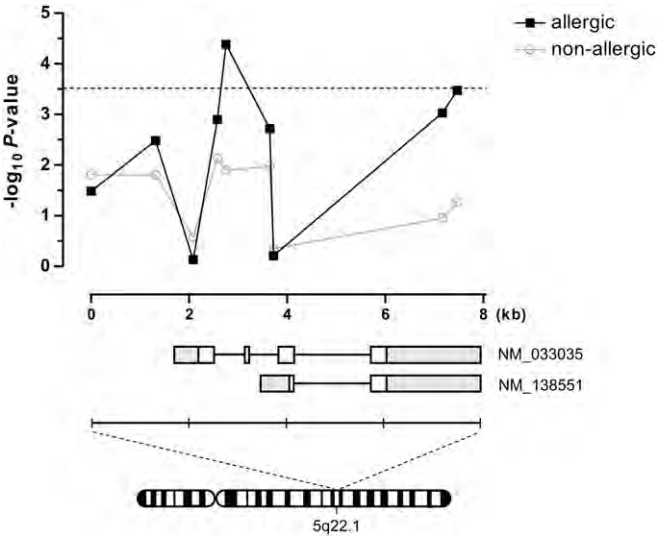

FIG 1. Associations of *TSLP* SNPs with EE are independent of allergy phenotypes. *Upper panel*, The associated $-\log_{10}$ *P* values for the genotyped *TSLP* SNPs from each analysis (patients with EE vs allergic or nonallergic control subjects) are plotted based on relative base pair location. The *dashed line* represents the Bonferroni threshold for significance ($P = 3 \times 10^{-4}$). *Lower panel*, The *TSLP* SNPs reside within an approximate 8-kb interval on 5q22.1 encoding the *TSLP* gene isoforms (NM_ 033035 and NM_138551). Represented are exons *(white boxes)*, introns *(bold lines)*, and untranslated regions *(gray boxes)*.

RESULTS

In our effort to identify EE-specific genetic susceptibility loci, we used a custom Illumina SNP genotyping chip to screen for variants within candidate genes involved in allergy, epithelial cell function, or both. The Bonferroni LD-adjusted *P* value required for statistical significance in this study was determined to be 3×10^{-4} to account for multiple testing. Because the coincidence of allergy is high (approximately 70%) in patients with EE, we first chose to investigate the specificity of any potential associations of candidate gene SNPs by stratifying the discovery control cohort into 2 phenotypically defined groups based on a history of allergy (see the Methods section). When comparing patients with EE with the allergic control subjects, we found that only *TSLP* harbored variants that reached statistical significance (Table I and Fig 1 and see Table E1 in this article's Online Repository at www.jacionline.org). Indeed, 7 SNPs in *TSLP* exhibited association with EE ($4.11 \times 10^{-5} \leq P \leq 3.29 \times 10^{-3}$). However, when comparing patients with EE with the nonallergic control subjects, no genes exhibited variants that reached statistical significance; the strongest observed *TSLP* SNP association only reached a *P* value of 7.35×10^{-3} in this analysis. When the 2 control groups were combined (allergic and nonallergic), the association strengthened, most likely because of the increase in the sample size of the control group. Interestingly, polymorphisms in *IL4*, a known allergy susceptibility gene, were associated with EE (best $P = 1.33 \times 10^{-3}$; OR, 1.91) using the nonallergic control

TABLE II. Replication of *TSLP* SNP association with EE in 2 independent case-control cohorts

	Discovery cohort*				Replication cohort†				
SNP	MAF cases	MAF control subjects	OR	*P* value	MAF cases	MAF control subjects	OR	*P* value	Combined *P* value‡
rs3806933	0.31	0.40	0.66	2.38E-03	0.31	0.41	0.66	6.10E-02	1.43E-03
rs2289276	0.24	0.26	0.86	3.43E-01	0.26	0.23	1.17	5.01E-01	4.74E-01
rs10062929	0.08	0.15	0.45	5.79E-04	0.09	0.19	0.36	3.31E-04	3.16E-06
rs2289277	0.31	0.41	0.64	1.35E-03	0.36	0.43	0.75	1.69E-01	2.14E-03
rs11466749	0.11	0.17	0.52	3.08E-03	0.11	0.21	0.46	5.00E-03	1.86E-04
rs11466750	0.09	0.15	0.70	9.02E-03	0.10	0.19	0.46	9.00E-03	8.48E-04

*The discovery cohort was composed of 170 patients with EE and 466 allergic and nonallergic control subjects, as described in Table I.
†The replication cohort was composed of either 87 patients with EE and 114 allergic and nonallergic control subjects or 87 patients with EE and 122 allergic and nonallergic control subjects for SNP rs10062929.
‡Combined *P* values from the discovery and replication cohorts using the Fisher method.

subjects but not the allergic control subjects (best $P = .27$; see Table E1), emphasizing the importance of using phenotypically matched control subjects for identifying risk variants associated with primary disease. Moreover, these data highlight the specificity of the *TSLP* genetic association with EE.

We next aimed to replicate the observed genetic association with *TSLP* variants using a completely independent replication cohort of patients with EE and control subjects (Table II). Although the replication control cohort was composed of both allergic and nonallergic subjects, 3 *TSLP* SNPs were significantly associated with EE (Table II, $P < .5$) in this independent analysis. In a meta-analysis of the associated *TSLP* variants from both the discovery and replication cohorts, the association of rs10062929 strengthened (combined $P = 3.16 \times 10^{-6}$) approximately 2 logs above the threshold of the Bonferroni adjustment. Moreover, the combined *P* values for rs11466750 and rs11466749 were nominally significant (combined $P = 8.48 \times 10^{-4}$ and 1.86×10^{-3}, respectively).

The prevalence of EE in male subjects is approximately 2.5-fold higher than in female subjects. To determine whether *TSLP* variants contribute to this sex bias, we performed a sex-stratified analysis of patients with EE and allergic control subjects from the discovery cohort and determined the SNPs with the greatest change in *P* value in either the male or female cohorts (see Table E2 in this article's Online Repository at www.jacionline.org). Of the 9 *TSLP* SNPs, only rs10062929 and rs11466749 also associated in the female EE cohort ($P = .016$ and $P = .048$ in patients with EE vs female atopic control subjects, respectively), whereas the *P* values of most variants in *TSLP* remained unchanged in the male-only analyses.

Interestingly, *TSLPR* is encoded on a pseudoautosomal region on Xp22.3 and Yp11.3, which further underscores the potential significance of finding SNPs in the *TSLP/TSLPR* pathway observed among male patients with EE.[17] We were interested in testing the hypothesis that variants in *TSLPR* might also associate with EE in a sex-specific manner. Therefore we genotyped 3 coding SNPs recently validated from direct sequencing of the *TSLPR* gene (P.G., unpublished data): rs36139698, rs36177645, and rs36133495. Of these, the SNP rs36133495 (Ala to Val) was significantly associated with disease risk in a cohort of male patients with EE and healthy male control subjects (Val/Val: 27% in male patients with EE vs 15% in male control subjects; $P = .039$; OR, 2.05; Fig 2 and Table III). Conversely, rs36133495 was not significantly associated in a female case-control analysis ($P = .929$; OR, 1.22). Taken together, these data present evidence that the *TSLP* signaling pathway might contribute to the male bias seen in patients with EE.

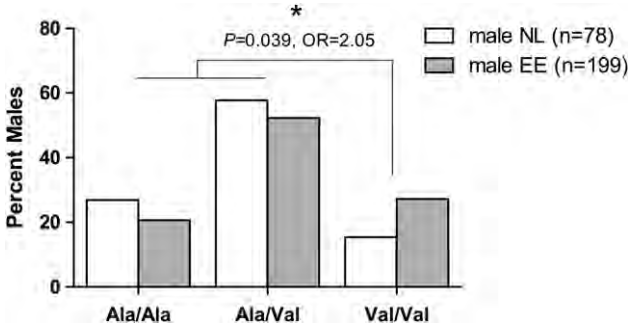

FIG 2. A nonsynonymous SNP in *TSLPR* associates with EE in a sex-specific manner. A nonsynonymous SNP (rs36133495) in *TSLPR*, which results in an Ala to Val coding change, shows a sex-based association with increased disease risk in male patients with EE, with Val/Val and Ala/Val subjects at higher risk (OR, 2.05) compared with Ala/Ala subjects. Genotyped were 199 male patients with EE and 78 male healthy control subjects *(NL)*.

In the lung and skin TSLP is produced primarily by epithelial cells in response to T_H2 cytokines or TLR3 agonists, subsequently targeting dendritic cells to develop T_H2-polarizing activity, including cytokine and chemokine production[13,18]; however, it has been observed that activated mast cells can also express *TSLP* to similarly drive T_H2 responses.[12,19,20] Indeed, we have recently observed increased expression of *TSLP* mRNA in the esophagi of patients with EE compared with that seen in control subjects.[10] Accordingly, we aimed to determine whether primary esophageal epithelial cells could produce TSLP. Notably, exposure to the TLR3 ligand poly I:C (a double-stranded RNA mimetic) robustly increased *TSLP* mRNA expression in as little as 3 hours after treatment with either 10 or 100 μg/mL poly I:C (Fig 3). These data suggest that the esophageal epithelium is a source of *TSLP* expression in patients with EE.

DISCUSSION

Herein we report that *TSLP* is the most dominant genetic variant associated with EE risk using a large panel of SNPs within relevant allergy and epithelial gene products. We demonstrate that the genetic association of EE with *TSLP* occurs largely independent of allergy, providing compelling evidence in support of our recent GWAS analysis that identified the 5q22 locus as a risk locus for EE susceptibility.[10] It is notable that the direction of the disease risk (OR < 1) was similar in the discovery and replication cohorts in our present study and recent EE GWAS, supporting the genetic association to this region. However, given that the

TABLE III. A sex-specific association of a *TSLPR* SNP in male patients with EE

	Male patients with EE (n = 199), male control subjects (n = 78)				Female patients with EE (n = 105), female control subject (n = 78)			
SNP*	MAF cases	MAF control subjects	OR	*P* value†	MAF cases	MAF control subjects	OR	*P* value†
rs36133495	0.53	0.44	2.05	.039	0.47	0.42	1.22	.929

*dbSNP Build 130 rs number.
†*P* value using a dominant model of association.

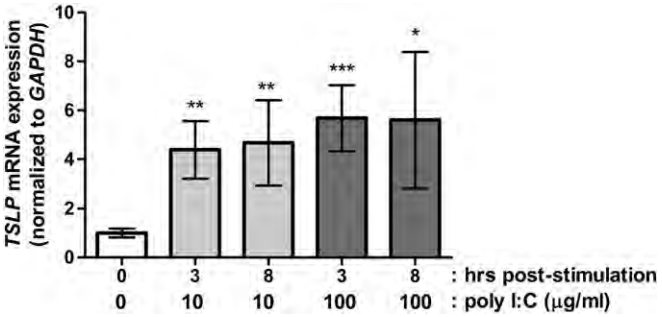

FIG 3. TLR3 signaling stimulates *TSLP* expression in primary esophageal epithelial cells. Treatment of primary esophageal epithelial cells with the TLR3 agonist poly I:C induces a robust increase in *TSLP* mRNA levels. Data shown are the means ± SEMs from 4 to 5 independent experiments performed in duplicate. *$P < .05$, **$P < .01$, and ***$P < .001$. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

more common allele is associated with disease susceptibility, the causative allele in EE is unlikely to have been identified. That the same genetic region (5q22) has been linked to blood eosinophilia further implicates a potential relationship between *TSLP* and eosinophilic disease.[21]

In exploring potential mechanisms for the male predilection observed in the population with EE, we performed a sex-stratified analysis for *TSLP* and *TSLPR* polymorphisms. Indeed, a sex-specific interaction was supported for *TSLP* variants, as well as a nonsynonymous SNP within *TSLPR* on Xp22.3/Yp11.3 in male patients with EE. Bioinformatic analysis of the variant suggests that the mutant *TSLPR* has increased stability compared with the wild-type protein, which could conceivably prolong *TSLP*-induced signal transduction.[22] In a recent study by Mullighan et al,[23] comparative genomic hybridization analysis in patients with B-progenitor acute lymphoblastic leukemia identified a fusion event between exon 1 of the P2RY8 receptor and *TSLPR*, resulting in a constitutive activation of the Janus kinase–signal transducer and activator of transcription pathway, indicating the profound ability of the *TSLP* pathway to induce disease.

We also demonstrate that *TSLP* mRNA expression is induced in primary esophageal epithelial cells after activation of the TLR3 pathway; it is notable that viral gastroenteritis–like symptoms often precede the onset of EE. *TSLP* was originally identified as a prosurvival factor that could induce pre–B-cell differentiation and proliferation.[24] Constitutive phosphorylation of signal transducer and activator of transcription 5 has been linked with lymphoproliferative diseases and malignancies.[25] Perhaps *TSLP* overexpression could contribute to both the inflammatory and hyperproliferative states observed in the esophageal epithelium of patients with EE.

Beyond elucidating a role for *TSLP* in patients with EE, these results have broad implications for the discovery of rare disease risk variants. Specifically, these results demonstrate the importance of appropriate control cohort selection. Consideration of comorbid diseases might provide insight into the role of genetic risk variants, particularly when a rare disease phenotype (EE) tracks with a more common phenotype (allergy). In the present study we show that use of well-characterized control populations in genetic association studies can overcome relatively small sample sizes to identify true risk variants.

In conclusion, we have identified a specific genetic contribution of *TSLP* to EE susceptibility, a finding that provides key insight into disease pathogenesis and likely explains, at least in part, the male sex bias in this disease. We propose that activation of the innate immune system in the esophageal epithelium involving *TSLP* is likely to have a key role in the subsequent adaptive allergic response ultimately triggered by food antigens. These findings present *TSLP* as a potential molecular target for therapeutic intervention and thus highlight the key role of innate immunity in the development of specific allergic disease.

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Key messages

- **Polymorphisms in *TSLP* are risk factors for EE independent of underlying allergy phenotypes.**
- **A sex-specific association between SNPs in *TSLP*, as well as a nonsynonymous SNP in *TSLPR*, suggests a mechanism for the male predilection of EE.**
- **Primary esophageal epithelial cells express *TSLP* mRNA in response to TLR3 signaling.**

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ALLERGY ARCHIVES

THE EOSINOPHIL, A HISTORICAL RETROSPECTIVE: TOXIC FUNCTION

Gerald J. Gleich, MD (provided by Dr G. J. Gleich).

The question of whether blood and tissue eosinophils characterizing human disease function to the benefit or detriment of the host was addressed in the investigations of Gleich (1931-) at the Mayo Clinic. In 1973, he identified, isolated, and characterized the major basic protein (MBP) in the crystalline core of the guinea pig eosinophil granule.[1] Demonstration of MBP as the eosinophil-derived agent that damaged tegument of parasitic helminths and murine ascites tumor cells raised the possibility that it might damage cells from organs infiltrated with eosinophils. Gleich's extended studies centered on determining whether MBP could damage normally functioning cells and whether concentrations of MBP in the toxic range were present in fluids bathing organs.

Findings indicated that MBP damaged cells from organs whose dysfunction was frequently associated with an infiltration of eosinophils.[2] *In vitro* injurious damaging concentrations present in body fluids from patients with eosinophil-associated disorders pointed to the possibility that the eosinophil may impair organ function in disease states. That concept could explain positive associations between eosinophilia and organ dysfunction in asthma, vasculitis, and hypereosinophilic syndrome and beneficial effects of glucocorticoids, which at therapeutic doses reduced eosinophil numbers in blood and presumably in tissues.

At about the same time, Austen and his associates were studying modulating effects of eosinophil granular enzymes on allergic reactions. To read about their investigative findings, please visit www.jacionline.org/content/allergy_archives for an Allergy Archive summary entitled "Immune and Hypersensitivity Relevance."

1. Gleich GJ, Leogering DA, Maldonato GJ. Identification of a major basic protein in guinea pig eosinophil granules. J Exp Med 1973;137:1459-71.

2. Gleich GJ, Evangelo F, Loegering DA, Wassom DL, Steinmuller D. Cytotoxic properties of the eosinophil major basic protein. J Immunol 1979;123:2925-7.

METHODS

Statistical analysis

Quality control filtering and adjustment for population stratification were performed before sex stratification, as described. To test for genotype-by-sex interaction, we first performed split-sample analysis, in which the male and female subjects were run separately to determine whether the association was strong in one of the sexes. We then performed formal genotype-by-sex interaction analysis in PLINK to ensure that different strengths of association were not due to differences in sample size.

TABLE E1. Top 10 SNPs most associated with EE in discovery cohort analysis

Patients with EE (n = 170) vs allergic control subjects (n = 220)

	Gene	CHR	SNP*	BP†	Minor allele	MAF cases	MAF control subjects	OR	*P* value
1	*TSLP*	5	rs10062929	110436078	A	0.08	0.17	0.35	4.11E-05
2	*TSLP*	5	rs11466750	110440793	A	0.09	0.18	0.43	3.37E-04
3	*TSLP*	5	rs11466749	110440484	G	0.11	0.19	0.48	9.29E-04
4	*TSLP*	5	rs1898671	110435901	T	0.45	0.33	1.61	1.26E-03
5	*TSLP*	5	rs2289277	110436966	G	0.31	0.42	0.61	1.90E-03
6	*TSLP*	5	rs3806933	110434641	T	0.31	0.41	0.63	3.29E-03
7	*IL10*	1	rs3024500	205007454	C	0.41	0.50	0.65	5.99E-03
8	*IL10*	1	rs2222202	205012004	T	0.41	0.50	0.66	8.06E-03
9	*DNAH5*	5	rs11958133	13794223	C	0.26	0.18	1.60	8.79E-03
10	*IL10*	1	rs3024496	205008487	C	0.41	0.50	0.67	1.07E-02

Patients with EE (n = 170) vs nonallergic control subjects (n = 246)

	Gene	CHR	SNP	BP	Minor allele	MAF cases	MAF control subjects	OR	*P* value
1	*IL4*	5	rs2243250	132037053	T	0.18	0.10	1.91	1.33E-03
2	*IL4*	5	rs2243282	132044453	A	0.18	0.10	1.79	3.96E-03
3	*IL4*	5	rs2243268	132041862	C	0.17	0.10	1.77	5.09E-03
4	*KIF3A*	5	rs3798130	132070045	A	0.17	0.10	1.76	5.47E-03
5	*TSLP*	5	rs1898671	110435901	T	0.45	0.35	1.48	7.35E-03
6	*KIF3A*	5	rs2299011	132070441	G	0.17	0.10	1.71	9.07E-03
7	*TSLP*	5	rs2289277	110436966	G	0.31	0.40	0.69	1.05E-02
8	*IL4*	5	rs2243274	132042731	A	0.18	0.11	1.66	1.08E-02
9	*ADCY2*	5	rs12652807	7502380	G	0.09	0.14	0.55	1.11E-02
10	*IL10*	1	rs3024500	205007454	C	0.41	0.50	0.69	1.15E-02

Patients with EE (n = 170) vs combined control subjects (n = 466)

	Gene	CHR	SNP	BP	Minor allele	MAF cases	MAF control subjects	OR	*P* value
1	*TSLP*	5	rs1898671	110435901	T	0.4471	0.3411	1.57	5.52E-04
2	*TSLP*	5	rs10062929	110436078	A	0.07647	0.1516	0.45	5.79E-04
3	*TSLP*	5	rs2289277	110436966	G	0.3118	0.4093	0.64	1.35E-03
4	*TSLP*	5	rs3806933	110434641	T	0.3107	0.403	0.66	2.38E-03
5	*TSLP*	5	rs11466750	110440793	A	0.08929	0.1544	0.52	3.08E-03
6	*IL10*	1	rs3024500	205007454	C	0.1765	0.1753	0.68	3.85E-03
7	*IL10*	1	rs2222202	205012004	T	0.4118	0.4978	0.70	7.37E-03
8	*IL10*	1	rs3024496	205008487	C	0.4118	0.4946	0.70	8.47E-03
9	*TSLP*	5	rs11466749	110440484	G	0.1088	0.1688	0.59	8.47E-03
10	*TSLP*	5	rs3806932	110433574	G	0.3254	0.4056	0.70	9.02E-03

CHR, Chromosome.

*dbSNP Build 130 rs number.

†*BP*, Base pair; chromosomal location National Center for Biotechnology Information (NCBI) Build 36.

TABLE E2. Sex stratification for *TSLP* risk variants

		Female sex*		Male sex†	
SNP	**BP**	**OR**	***P* value**	**OR**	***P* value**
rs10062929	110436078	0.34	1.57E-02	0.35	9.50E-04
rs11466750	110440793	0.48	6.84E-02	0.38	1.69E-03
rs11466749	110440484	0.48	4.78E-02	0.48	1.02E-02
rs1898671	110435901	1.36	2.08E-01	1.77	3.40E-03
rs2289277	110436966	0.62	7.62E-02	0.59	1.14E-02
rs3806933	110434641	0.66	1.23E-01	0.61	1.60E-02

*Female patients with EE (n = 53); female allergic control subjects (n = 94).
†Male patients with EE (n = 117); male allergic control subjects (n = 126).

Appendix 3.

Series editors: Joshua A. Boyce, MD, Fred Finkelman, MD, William T. Shearer, MD, PhD, and Donata Vercelli, MD

Genetic dissection of eosinophilic esophagitis provides insight into disease pathogenesis and treatment strategies

Joseph D. Sherrill, PhD, and Marc E. Rothenberg, MD, PhD *Cincinnati, Ohio*

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List of Design Committee Members: Joseph D. Sherrill, PhD, and Marc E. Rothenberg, MD, PhD

Activity Objectives

1. To identify the epidemiology of eosinophilic esophagitis (EoE).
2. To distinguish EoE from gastroesophageal reflux disease (GERD).
3. To apply knowledge of susceptibility to clinical cases.
4. To apply genetic knowledge to emerging diagnostics and therapeutics.
5. To integrate results from genetic susceptibility loci studies with gene expression changes in patients with EoE.

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Eosinophilic esophagitis (EoE) is a chronic inflammatory disorder of the esophagus that is compounded by both genetic predisposition and aberrant responses to environmental antigens, particularly those that are food derived. Data have indicated a unique transcriptional response *in vivo* that defines EoE and that appears to be partially attributable to the T_H2 cytokine IL-13. Moreover, a number of genetic risk variants in proinflammatory and epithelial cell genes associate with EoE susceptibility, demonstrating novel heritable mechanisms that contribute to disease risk. Here we discuss recent advances in our understanding of the intrinsic (genetic) and extrinsic (environmental) components that illustrate the complex nature of EoE. (J Allergy Clin Immunol 2011;128:23-32.)

Key words: *Eosinophilic esophagitis, genetics, candidate gene, genome-wide association, polymorphism*

The phenomenon of esophageal eosinophilia can be traced through the published literature as far back as 1962.[1] Since these early reports, much progress has been made at the molecular and clinical levels to tease apart the intricacies that distinguish eosinophilic esophagitis (EoE) from other inflammatory disorders, including gastroesophageal reflux disease (GERD). As the prevalence of these diseases has increased since the late 1990s, the need for improved diagnoses, both from a therapeutic and research standpoint, has arisen. Moreover, the high degree of overlap in presenting symptoms in EoE and GERD have been problematic clinically and have necessitated the establishment of diagnostic criteria to differentiate the 2 diseases.

In 2007, the First International Gastrointestinal Research Symposium published initial guidelines for the clinical diagnosis of EoE based on the symptomatology and histology of the disease.[2] More recently, updated consensus recommendations from a panel of allergists, pathologists, and gastroenterologists have been stated (see Liacouras et al[3] in this issue of the *Journal*). These recommendations emphasize that EoE is a chronic,

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Terms in boldface and italics are defined in the glossary on page 24.

Abbreviations used
AD: Atopic dermatitis
CRLF2: TSLP receptor singe nucleotide polymorphism
DSG1: Desmoglein-1 gene
EDC: Epidermal differentiation complex
EoE: Eosinophilic esophagitis
FLG: Filaggrin gene
GERD: Gastroesophageal reflux disease
GWAS: Genome-wide association study
hpf: High-powered field
POSTN: Periostin gene
SNP: Single nucleotide polymorphism
TSLP: Thymic stromal lymphopoietin
WDR36: WD repeat domain 36 gene

antigen-driven clinicohistopathological disorder that is causatively and epidemiologically distinct from GERD. Clinically, EoE is characterized by a spectrum of symptoms indicative of esophageal dysfunction. Pathologically, 1 or more esophageal mucosal biopsy specimens show eosinophil-predominant inflammation in excess of 15 intraepithelial eosinophils per high-powered field (hpf). The disease is isolated to the esophagus, and other causes of esophageal eosinophilia should be excluded. The peak age of EoE diagnosis occurs within the first 3 years of life,[4] most likely resulting from antigen hypersensitivity as solid foods are introduced, although diagnosis in adults is also common. Disease remission typically occurs with treatment, which might include dietary exclusion, topical corticosteroids, or both.[3] The symptomatology of EoE, if untreated, follows a trend according to patient age. In pediatric patients, these symptoms begin as difficulty feeding and vomiting and can result in failure to thrive.[5,6] In adolescent and adult patients, abdominal pain, dysphagia, and food impaction are the chief presentations of the disease.[6,7] Endoscopic examination has identified common esophageal abnormalities associated with EoE, such as ***linear furrowing*** with loss of vascularity, ring-like structures, and the presence of ***white exudate*** on the esophageal epithelium.[2,8] Histologically, the esophageal epithelium exhibits extensive ***basal zone hyperplasia*** with ***papillary elongation*** and fibrosis within the lamina propria and accumulation of eosinophils, B lymphocytes,[9] $CD4^+$ and $CD8^+$ T lymphocytes,[10] regulatory T cells,[11] and mast cells.[12-14]

In addition to being resistant to acid neutralization therapy, another distinguishing feature of EoE is the high rate of concurrent atopy. Studies have indicated a prominent role for food allergies in patients with EoE, with published frequencies ranging from 46% to 79% within the EoE population.[4,5,15,16] In comparison with the estimated 22% of patients with peanut allergy who have tolerance later in life,[17] only a very small percentage (<10%) of patients with EoE have tolerance to food antigens, as defined by sustained disease remission,[15] demonstrating the

GLOSSARY

BASAL ZONE HYPERPLASIA, PAPILLARY ELONGATION: Histologic findings in patients with EoE that are caused by active basal cell proliferation and increased extension of the vascular papillae and subepithelial lamina propria into the epithelial space. Other typical histologic features of EoE include dilated intercellular spaces and lamina propria fibrosis.

CCR3, EOTAXINS: CCR3 binds eotaxins. Although eotaxin-1 and eotaxin-2 target eosinophils to the lung and lower gastrointestinal tract, eotaxin-3 is present only in human subjects and functions as a chemoattractant for esophageal eosinophils.

CHROMATIN IMMUNOPRECIPITATION (CHIP): ChIP technology uses antibodies to precipitate a protein bound to DNA. The bound DNA sequence can be analyzed to look for target sequences for transcription factors or histone-associated regions of DNA.

EPIGENETICS: The study of changes in DNA configuration that allow for changes in gene expression independent of sequence changes. For example, DNA methylation can cause DNA closing, making specific genetic regions inaccessible to RNA polymerase and transcription factors, thus silencing gene expression. Histone acetylation can allow DNA to open and can increase transcription. CREB-binding protein (CBP, p300) is a histone acetylase implicated in EoE pathogenesis.

FILAGGRIN, INVOLUCRIN: Involucrin is present in the cytoplasm of keratinocytes and is cross linked to cell membrane proteins through transglutaminase. This allows the formation of a strong epithelial barrier and decreases skin invasion by microorganisms. The usual function of filaggrin is to function as a natural moisturizing factor. Loss of function in the filaggrin gene causes ichthyosis vulgaris and predisposes to eczema, asthma, and EoE.

GENOME-WIDE ASSOCIATION STUDY (GWAS): GWASs use gene chip technology and bioinformatics to analyze the human genome for SNPs in diseased and nondiseased states. Haplotypes (in blocks) that vary between diseased and nondiseased subjects are considered to be associated with the disease state.

IL-13: IL-13 is a T_H2 cell–derived interleukin capable of inducing multiple aspects of eosinophil-associated tissue remodeling. IL-13 overexpression in target organs of transgenic animals is associated with pulmonary, esophageal, and cutaneous fibrosis, as well as angiogenesis.

LAMBDA (λ): The familial relative risk is defined by the statistic λ, which assesses the risk of disease in a subject with a diseased first-degree biological relative to the risk in the population at large. The larger the λ value, the stronger the genetic effect is on the disease.

LINEAR FURROWING, WHITE EXUDATES: Typical endoscopic findings in patients with EoE include esophageal lichenification, linear furrowing, pallor, and white exudates/plaques (histologically comprised of eosinophils), strictures, and concentric rings caused by motility or fibrosis.

SINGLE NUCLEOTIDE POLYMORPHISM (SNP): Genetic variant in a single nucleotide that might be normally present in the population and associated with risk for certain complex polygenic diseases.

THYMUS AND ACTIVATION-REGULATED CHEMOKINE (TARC): TARC, also known as CCL17, induces migration of $CCR4^+$ T cells to the skin in patients with eczema, and its expression can be increased in the periphery of some patients with EoE.

TRANSCRIPTOMES, PROTEOMES: Gene expression microarray defines transcriptional differences among subjects with and without a disease state. Gene chip technology and bioinformatics are used to analyze the level of gene expression (referred to as the "transcriptome") across the genome in diseased versus control populations. Proteomes are the protein expression pattern of an organism. Proteomic analysis can use techniques, such as 2-dimensional gel analysis and peptide sequencing or antibody arrays, to find both previously known and unknown proteins associated with a particular disease state.

TGF-β: TGF-β is produced by epithelial cells and inflammatory cells, including eosinophils, and mast cells and has profibrotic effects. TGF-β1, 2, and 3 reside on distinct chromosomes but use the same signaling pathway and receptors.

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chronic nature of EoE. The most effective therapies currently used to manage EoE are food antigen avoidance or swallowed glucocorticoid treatment.[18] Although these treatments can reduce or eliminate disease symptoms, relapse commonly occurs after reintroduction of allergens or discontinuation of treatment, suggesting that food antigen hypersensitivity is a fundamental feature of EoE. A number of empiric and test-based food elimination trials have further implicated food hypersensitivity in the population with EoE; however, the high variability and low predictive value of skin prick tests and serum IgE measurements as demonstrated in these studies suggest that the clinical utility for standardized assessment for food-specific reactivity in patients with EoE remains to be determined (see Chehade and Aceves[16] for a thorough review of clinical food trials in patients with EoE). Other atopic diseases, such as atopic dermatitis (AD), asthma, and allergic rhinitis, are also common in the population with EoE.[5] Although these diseases are largely mediated by enhanced sensitivity to aeroallergens, the exacerbated T_H2 inflammation and tissue remodeling that occurs within the affected tissues indicate shared mechanisms of disease with EoE.

EPIDEMIOLOGY

With the expansion of EoE cases reported worldwide, multiple studies have aimed to establish a baseline prevalence of EoE and determine whether disease incidence has increased. From 2000-2003, the estimated prevalence of EoE in a pediatric population was approximately 4 in 10,000, with an incidence rate of 0.9 to 1.3 in 10,000 new cases per year.[5] A similar prevalence (approximately 2 cases per 10,000) and incidence rate (1.4 per 100,000) during a 16-year period were observed in an adult Swiss cohort.[19] A study of 1000 esophageal biopsy specimens from a randomized Swedish cohort showed a disease prevalence of 0.4%, as defined by using an esophageal eosinophil level of greater than 20/hpf.[20] However, in a retrospective study examining esophageal biopsy specimens from 666 pediatric patients given diagnoses of esophagitis from 1982-1999, data suggest that although the prevalence of EoE was increasing, the incidence of disease was relatively stable, despite the marked increase in esophagogastroduodenoscopies during that time period.[21] By using the current recommended eosinophil threshold for EoE diagnosis (>15/hpf), 198 of these patients had sufficient eosinophils levels, with many having histologic indications of basal layer expansion and lamina propria fibrosis to indicate a retrospective diagnosis of EoE.[21] These findings suggest that enhanced disease recognition, rather than a true increase in disease incidence, underlies the emergence of EoE within the last decade. Notably, dysphagia was significantly associated with retrospective EoE, and the ancestry and sex of these cases were similar to those currently reported, with the majority being white (81%) and male (72%).[21]

An interesting questionnaire-based study on the geographic distribution of EoE across the United States has indicated higher disease prevalence in urbanized areas, with a higher concentration of EoE observed in the northeastern states. Here the estimated prevalence of EoE in the entire United States was 52 per 100,000.[22] A similar trend of a higher EoE prevalence in urban areas was shown to be independent of race, indicating that environment has an equally important contribution as genetics to EoE risk.[23] Certainly the high incidence of asthma among urban populations, as demonstrated by multiple groups, has garnered significant attention[24] and given credence to the general hypothesis that increased exposure to aeroallergens is a predisposing factor. The findings by Spergel et al[22] and Franciosi et al,[23] which define these EoE "hot zones" within urban settings, implicate a similar effect of socioeconomic factors in EoE susceptibility.

GENETIC HERITABILITY

An underlying genetic predisposition to EoE has been proposed by multiple groups that show a disproportionate prevalence of disease in white subjects and male subjects and within families of affected subjects.[15,23] For instance, data over a 14-year period demonstrated that 90% of the patients with EoE were white and 75% were male.[15] Reports of a familial occurrence of EoE and esophageal dilatation in 6.8% and 9.7% of patients with EoE, respectively, suggest that the prevalence of EoE and associated esophageal dysfunction is high among related subjects.[5] Furthermore, the increased risk of EoE among siblings is dramatic when compared with other disorders. For instance, the estimated sibling recurrence risk among siblings of patients with EoE ($\lambda_s = \sim 80$) is markedly higher compared with that of siblings with other atopic diseases with familial inheritance patterns, such as asthma ($\lambda_s = \sim 2$).[25] However, despite this strong familial inheritance, comparison of familial with sporadic cases of EoE showed no difference in esophageal pathology (with the exception of linear furrowing) and gene expression profiles.[26] Nonetheless, genetic predisposition and family history likely have a significant role in EoE susceptibility, and thus detailed family histories are paramount when encountering these patients.

TRANSCRIPTOME ANALYSIS

A major step toward the molecular mapping of EoE was achieved when gene expression profiling of patients' esophageal biopsy specimens showed a remarkable transcript signature that distinguishes patients with EoE from healthy control subjects and patients with chronic esophagitis.[27] Altered expression of approximately 574 genes comprises this EoE "***transcriptome***," which exhibits a high level of conservation among patients' sex, age, and atopic history and strongly correlates with esophageal eosinophil levels. The most highly induced gene in the esophagi of patients with EoE is the eosinophil chemoattractant ***eotaxin***-3 *(CCL26)*, which was overexpressed 53-fold in esophageal biopsy specimens from patients with EoE compared with normal esophageal biopsy specimens.[27] Eotaxin-3 belongs to the eotaxin family (eotaxin-1 to eotaxin-3) of CC chemokines and, through its receptor, ***CCR3***, activates downstream G protein signaling to drive eosinophil chemotaxis and activation. Of the eotaxins, only *CCL26* is upregulated in patients with EoE, and its expression correlates with eosinophil (and mast cell) levels within esophageal biopsy specimens, indicating a specific contribution in the disease.[27] Notably, levels of *CCL26* transcript in a single biopsy specimen are highly sensitive (89%) in distinguishing EoE from control populations[28] despite the histological "patchiness" of EoE across multiple biopsy specimens. In fact, histological examination of at least 3 biopsy specimens is required to achieve similar diagnostic sensitivity.[2,3] Immunofluorescence and *in situ* hybridization studies on esophageal biopsy specimens identify the esophageal epithelium as the main source of eotaxin-3 production.[27] *In vivo* models of EoE further illustrate the crucial role of eotaxin-3 in disease because mice deficient in

the eotaxin receptor *Ccr3* are protected from esophageal eosinophilia after allergen challenge.[27] Steroid therapy, in particular swallowed glucocorticoids, effectively normalizes as much as 98% of the EoE transcriptome,[29] including *CCL26*, indicating the dynamic nature and reversibility of the genetic dysregulation.

In addition to eotaxin-3, a number of immune cell–specific genes exhibit differential expression levels in patients with EoE. For instance, expression of immunoglobulin genes and genes involved in antibody class switching is increased, reflecting the increase in the esophageal B-cell population in patients with EoE.[9] Mast cell–specific genes, specifically carboxypeptidase 3A *(CPA3)*, high-affinity IgE receptor *(FCERI)*, and tryptase-α *(TPSAB1)*, are abundantly represented in the EoE transcriptome, and mast cells are indeed a prominent inflammatory cell in the esophagi of patients with EoE when specifically examined with anti-tryptase staining.[12,27] Based on mast cell levels, a specific esophageal transcriptome is also identified in patients with EoE, which only partially overlaps with the transcriptome defined by eosinophil levels alone,[12] indicating that mast cells and eosinophils are likely independently involved, at least in part. Significant increases in mast cell degranulation and mastocytosis within the epithelium, lamina propria, and smooth muscle layer,[12,13] which can be ameliorated with steroid therapy,[12] further implicate these cells in the local inflammatory milieu within the esophagus.

A significant portion of the gene transcriptional changes associated with EoE occurs within the esophageal epithelium. These structural cells can influence multiple aspects of the disease phenotype, including inflammatory cell recruitment, tissue remodeling, and hyperproliferation. The human esophageal epithelium is composed of nonkeratinized, stratified squamous epithelia with a proliferating basal layer of 1 to 3 cells in depth and a differentiating suprabasal layer migrating toward the esophageal lumen.[30] Many of the histopathological features of the esophagus that are associated with EoE indicate gross defects in cell adherence, as indicated by dilated intercellular spaces, expansion of the basal cell layer, and extracellular matrix deposition within the lamina propria. Studies have highlighted ***IL-13*** as a critical signaling molecule capable of altering global gene expression of the esophageal epithelium. *Ex vivo* microarray analysis showed that treatment of biopsy-derived primary esophageal epithelial cells with IL-13, which is upregulated at the mRNA level in patients with EoE, can largely recapitulate the EoE transcriptome.[29] This study also confirmed epithelial cells as the primary source of *CCL26* in patients with EoE, which was upregulated by an astounding 279-fold after IL-13 stimulation *ex vivo*.[29] Notably, esophageal epithelial cells derived from patients with EoE and control subjects respond similarly to IL-13, as assessed based on *CCL26* production.[31]

Animal models have provided demonstrative data highlighting the robust proinflammatory action of IL-13 in an *in vivo* setting. Lung-specific overexpression of *Il13* in mice induces an asthma-like phenotype in the absence of antigen challenge that is characterized by marked inflammatory cell infiltration into the lungs and enhanced airway mucus production.[32] However, this model also promotes inflammation within the esophagus, such as esophageal eosinophilia and tissue remodeling, including fibrosis, angiogenesis, and epithelial hyperplasia.[33] The esophageal remodeling in this model occurs independently of eosinophilia and is inhibited by the type 2 IL-13 receptor (IL-13Rα2).[33] In summary, these findings implicate the esophageal epithelium as the pathogenic target of IL-13 signaling in patients with EoE, as demonstrated by the induction of pronounced histologic and molecular changes that occur in the presence of this potent T_H2 cytokine.

The epidermal differentiation complex (EDC) on human chromosome 1q21 is a cluster of genes that regulates terminal differentiation and formation of the cornified envelope of the epithelium.[34] Despite the lack of a cornified layer in the esophagus, the EDC locus contains the highest density of dysregulated genes in the EoE transcriptome compared with all other loci in the genome.[31] Loss-of-function mutations in several EDC genes, including ***filaggrin*** *(FLG)*, have been reported for various cutaneous disorders.[35-39] *FLG*, ***involucrin*** *(IVL)*, and several small proline-rich repeat (*SPRR*) family members (2C, 2D, and 3) are expressed in esophageal epithelial cells but are downregulated in response to IL-13 *ex vivo*,[31] implicating a homeostatic role for the EDC in the esophageal epithelium. Loss of *FLG* expression and subsequent defects in epidermal barrier function have been demonstrated in patients with AD,[40,41] which frequently co-occurs with EoE. However, no significant difference in *FLG* expression is observed between atopic and nonatopic patients with EoE,[31] suggesting an alternative function for filaggrin in regulating the epithelial structure within the human esophagus.

It is important to note that 2% of the EoE transcriptome is not reversible after disease remission induced by swallowed glucocorticoids.[29] Interestingly, these transcripts include genes that are involved in regulating homeostatic and pathogenic responses in the epithelium, such as cadherin-like 26 *(CDH26)*, uroplakin 1B *(UPK1B)*, periostin *(POSTN)*, and desmoglein-1 *(DSG1)*.[29] Desmoglein-1 is a transmembrane desmosomal cadherin component of desmosomes and facilitates the calcium-dependent homotypic interactions between adjacent cells that impart both structure and mechanical strength to the epithelia. Expression of *DSG1* is decreased in both glucocorticoid-treated and untreated patients with EoE (77% and 87%, respectively) compared with that seen in healthy control subjects. Desmoglein-1 is of particular importance because it is the target of multiple inherited and acquired cutaneous disorders. Pemphigus foliaceus and pemphigus vulgaris are autoimmune diseases in which autoantibodies targeting desmoglein-1 decrease cellular adhesion, resulting in epidermal blistering.[42] Notably, epithelial microabcesses exhibiting pronounced eosinophilic inflammation that can be associated with pemphigoid disorders have also been demonstrated within the esophagus, such as in pemphigus vegetans.[43] Furthermore, multiple heterozygous mutations in the extracellular domain coding region of *DSG1* have been linked with striate palmoplantar keratoderma, a disease characterized by epidermal thickening on the palms and soles.[44] Collectively, these findings substantiate the significance of alterations in desmoglein-1 in a spectrum of human diseases; it is tempting to speculate that tissue-specific decreases in desmoglein-1 might be pathogenic and partially responsible for the tissue-specific inflammation in patients with EoE.

POSTN is the gene of another key molecule that demonstrates steroid resistance in patients with EoE. Periostin, which functions as a cell adhesion molecule that regulates extracellular matrix deposition,[45,46] is dramatically upregulated in patients with EoE by approximately 52-fold; although glucocorticoid therapy can reduce a significant portion of this overexpression, *POSTN* expression remains increased in glucocorticoid-treated patients (approximately 2-fold).[47] Periostin is expressed in the basal epithelium and papillae[47] of the esophagus, suggesting a contributing role for the increased lamina propria fibrosis. Indeed, ***TGF-β***, a profibrotic stimulus that is expressed by eosinophils

and mast cells in biopsy specimens from patients with EoE,[13,48] can induce a dramatic upregulation of *POSTN* expression in primary esophageal fibroblasts, supporting this potential mechanism for the tissue fibrosis observed in patients with EoE.[47,49] Moreover, periostin can enhance eosinophil adhesion *in vitro*, and *Postn*-deficient mice are protected from allergen-induced eosinophilia in the lung and esophagus.[47] Interestingly, periostin upregulation in bronchial epithelial cells enhances TGF-β–induced collagen synthesis.[50] Because periostin also enhances cross-linking of collagen fibrils through upregulating the cleavage of mature active lysyl oxidase,[51] whose gene expression is also increased in the EoE transcriptome, these cumulative data suggest a positive feedback loop in which periostin has a central role in promoting the fibrotic responses in multiple inflammatory conditions.

In summary, esophageal transcript profiling has defined an EoE-specific transcript signature that is composed of dysregulated gene networks involved in T_H2 inflammation and epithelial cell responses. These studies demonstrate that IL-13 is a central mediator and link between the immunologic and histologic changes that are germane to EoE, largely through its effects on the esophageal epithelium. Given the well-documented role of IL-13 in other atopic diseases, such as asthma and AD, it is reasonable to speculate that IL-13 production in response to inhaled or absorbed antigens can also predispose subjects to other T_H2 comorbidities, such as EoE.

GENETIC VARIANTS AND DISEASE SUSCEPTIBILITY

The number of studies investigating genetic variants associated with EoE are few compared with those for other more common and more widely recognized atopic diseases, such as AD and asthma. Regardless, there have been significant strides in uncovering EoE risk variants in a relatively short period of time[14,27,52,53] due in part to the technological advances in genotyping ***single nucleotide polymorphisms*** (SNPs) in large case-control cohorts. In all, there have been 4 candidate gene studies that tested for polymorphisms in genes with a published (or suspected) functional role in EoE and one ***genome-wide association study*** (GWAS) that was used to identify EoE risk variants across the entire genome in an unbiased fashion (Table I).[14,27,31,52,53]

Candidate gene studies

Blanchard et al[27] identified the first EoE risk variant in a likely candidate, *CCL26*. The *CCL26* SNP (rs2302009) was shown to be highly associated with disease risk ($P = .001$), with an odds ratio of 4.55 in a case-control cohort. Transmission disequilibrium testing, which measures the transmission of a disease allele from unaffected heterozygous parents to an affected offspring, confirmed that the association of rs2302009 with EoE was not due to ancestral differences in the case-control analysis.[27] Additional studies have also linked this SNP to increased serum IgE levels and asthma susceptibility.[54] However, the observed association between rs2302009 and EoE was independent of atopic status, indicating a direct link with EoE susceptibility. Although rs2302009 is located within the 3′ untranslated region of the *CCL26* transcript and could potentially affect mRNA stability, a functional effect of this SNP in either asthma or EoE has yet to be described.

TGF-β, an eosinophil- and mast cell–derived mediator of fibrotic tissue responses, has been implicated in the same pathogenic process in patients with EoE.[48] Moreover, TGF-β1 has recently been shown to stimulate esophageal smooth muscle contractility and potentially contribute to esophageal dysmotility in patients with EoE.[13] An SNP within the *TGFB1* promoter (C-509T) that associated with asthma susceptibility[55,56] was shown to create a binding site for the transcription factor YY1 that subsequently enhanced promoter activity.[56] In a small cohort of 20 patients with EoE, homozygotes for the minor T allele of C-509T exhibited increased TGF-$\beta1^+$ lamina propria cell numbers.[14] Conversely, the major C allele of C-509T was a positive prognostic indicator for therapeutic responses in patients with EoE.[14] Determining the association of this and other *TGFB1* SNPs in a larger disease cohort will be vital to assess the full genetic contribution of *TGFB1* in patients with EoE.

Polymorphisms in epithelium-specific genes have also been associated with EoE susceptibility. First, a loss-of-function SNP in *FLG* (2282del4) that was previously linked with AD susceptibility[38] also associates with EoE risk; similar to the *CCL26* SNP, this association is specific to EoE because atopy was found not to be a confounding factor.[31] A second and larger candidate gene study examined 736 SNPs in 52 genes known to be involved in epithelial cell structure or inflammatory responses. Here, an EoE cohort of 170 patients was genotyped by using a custom SNP chip and compared with similarly genotyped control subjects with various atopic histories.[53] Importantly, SNPs in the gene for thymic stromal lymphopoietin *(TSLP)*, a cytokine recently described as a "master regulator" of T_H2 responses,[57] were shown to associate with EoE independent of the patient's atopic status. TSLP is derived primarily from epithelial cells in response to cytokines,[58] noxious substances,[59] and mechanical stress[60] and exerts its effects on nearly every cell type involved in T_H2 inflammation, including eosinophils[61] and mast cells.[62] For instance, TSLP activates dendritic cells to adopt a T_H2 priming phenotype through the secretion of the chemokines ***thymus and activation-regulated chemokine (TARC)***, macrophage-derived chemokine, and eotaxin-2 and OX40 ligand expression, which activates T_H2 cytokine production by naive $CD4^+$ T cells.[63-65] Thus, it is remarkable that TSLP critically regulates the exact processes involved in allergen sensitization that underscore the EoE phenotype. This study also identified an association between male patients with EoE and a nonsynonymous SNP in the TSLP receptor *(CRLF2)*,[53] which, given the male predilection for EoE, presents an intriguing scenario because *CRLF2* is encoded on pseudoautosomal region 1 of the X and Y chromosomes.[66]

EoE GWAS

A broader, unbiased GWAS approach was undertaken to identify SNPs associated with EoE susceptibility. Here, 2 relatively large cohorts of patients with EoE and healthy control subjects were genotyped for 550,000 SNPs across the genome.[52] Although only 1 locus on chromosome 5q22 was genome-wide significant after multiple testing correction, this region contains the genes encoding for *TSLP* and WD repeat domain 36 *(WDR36)*. Esophageal expression of *TSLP*, but not *WDR36*, is increased in patients with EoE, and the protective minor allele for the most significantly EoE-associated SNP on 5q22 (rs3806932), which lies upstream of the *TSLP* locus, correlates with decreased *TSLP* expression in the esophagus. Notably,

TABLE I. Genetic risk variants in patients with EoE

Chromosome	SNP*	Alleles†	Gene/gene locus	SNP location‡	Study design	*P* value and OR	Summary	Reference
7q11	rs2302009	T>G	Eotaxin-3 *(CCL26)*	3′ UTR	Candidate gene study (117 cases and 225 control subjects)	$P = .001$, OR = 4.55	Significance in a case-control association was also replicated by using transmission disequilibrium testing in a trio cohort.	Blanchard et al[27]
19q13	−(C-509T)	C>T	TGF-β1 *(TGFB1)*	Promoter	Candidate gene study (20 cases)	$P = .02$ for response status, $P = .01$ for TGF-$\beta1^+$ cells	CC genotype correlated with therapy response. The T allele was associated with increased numbers of TGF-$\beta1^+$ cells in lamina propria.	Aceves et al[14]
1q21	rs61816761 (2282del4)	CAGT>−	Filaggrin *(FLG)*	Exon	Candidate gene study (365 cases and 164 control subjects)	$P = .018$, OR = 4.89	Loss-of-function mutation in *FLG* associated with EoE	Blanchard et al[31]
5q22	rs10062929	C>A	*TSLP*	Intron	Large–scale candidate gene study (257 cases and 342 control subjects)	Meta-$P = 3.16 \times 10^{-6}$, OR = 0.36-0.45	*TSLP* SNPs associated with EoE risk independent of atopy	Sherrill et al[53]
Xp22/Yp11	rs36133495	G>T	TSLP receptor *(CRLF2)*	Exon	Candidate gene study (199 cases and 78 control subjects)	$P = .039$, OR = 2.05	Ala>Val amino acid change in TSLP receptor associated with male patients with EoE	Sherrill et al[53]
5q22	rs3806932 rs7723819	A>G G>A	*TSLP* *WDR36*	Near gene Near gene	GWAS (351 cases and 3104 control subjects)	Meta-$P = 3.19 \times 10^{-9}$, OR = 0.54-0.73 Meta-$P = 7.67 \times 10^{-9}$, OR = 0.55-0.71	Minor (protective) G allele correlated with decreased esophageal *TSLP* expression	Rothenberg et al[52]

OR, Odds ratio; *UTR*, untranslated region.
*dbSNP Build 131 "rs" identifier given when appropriate.
†Major allele > minor allele.
‡SNP location in relation to the gene/gene locus.

rs3806932 is in linkage disequilibrium with rs3806933,[52,67] suggesting that these 2 SNPs are inherited together more often than would be expected by chance. Data recently implicated rs3806933 in altering the binding of the transcription factor activator protein 1 to the *TSLP* promoter with a modest increase in promoter activity.[67] The other genome-wide significant SNP on 5q22 is upstream of the *WDR36* gene, which is located approximately 14 kb away from *TSLP* and lies within the same linkage block as rs3806932.[52] *WDR36* is critically involved in ribosomal RNA processing[68] and is coregulated with *IL2* in activated T lymphocytes.[69] Moreover, SNPs in the region of *WDR36* have been associated with peripheral blood eosinophilia,[70] as well as glaucoma susceptibility.[71] Thus, although *TSLP* appears to be the likely disease candidate on the 5q22 locus, the role of *WDR36* warrants further investigation.

GWASs on other more common gastrointestinal inflammatory diseases, such as Crohn disease,[72] ulcerative colitis,[73] and celiac disease,[74-76] have successfully identified numerous disease risk variants aided in part by the well-developed patient cohorts being historically investigated for these diseases. For example, meta-analyses across large, independent case-control cohorts (often in excess of 10,000 combined patients) along with further refinement of the human genome polymorphism map have yielded sufficient sample sizes to detect significant disease associations with common variants that have relatively low effect sizes. The low sample size of the current EoE GWAS (251 patients with EoE in total) not only emphasizes the magnitude of the 5q22 SNP associations but also suggests that there are likely additional EoE risk variants to be uncovered as further EoE cohorts are subjected to genome-wide genotyping and similar meta-analyses are

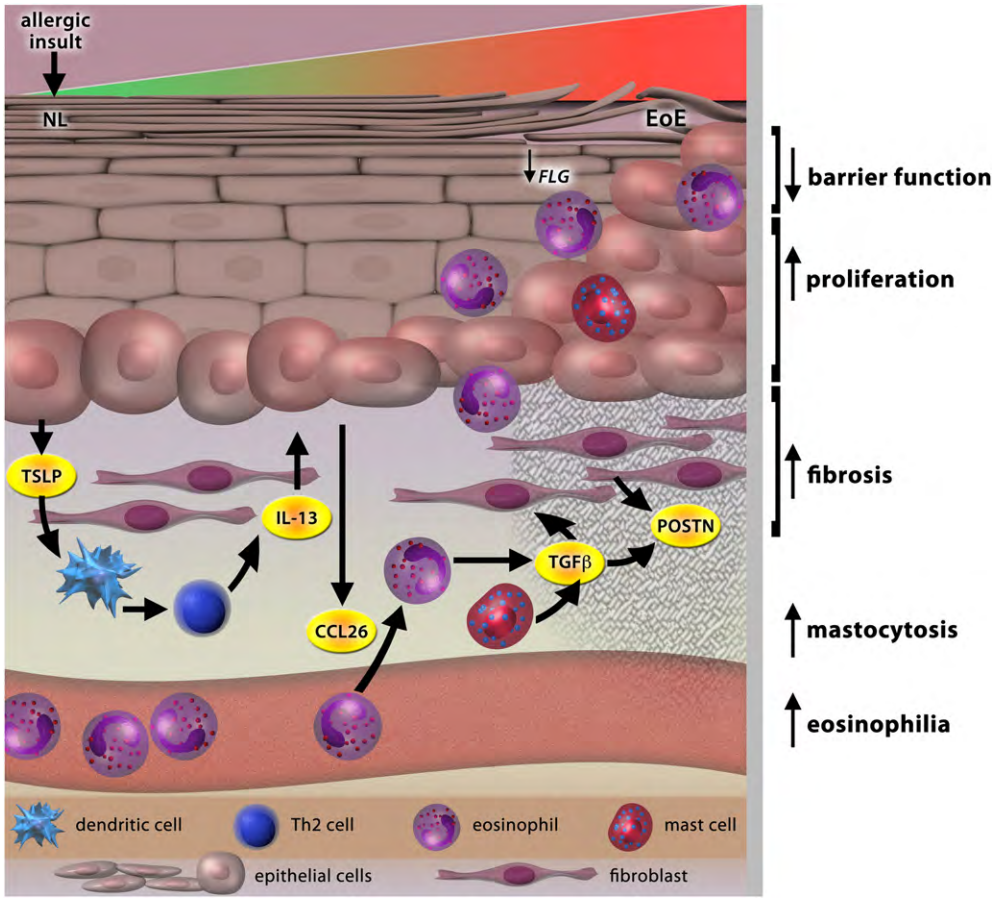

FIG 1. The molecular pathogenesis of EoE. An allergic insult by either food antigens or aeroallergens initiates the transition of the esophagus from a normal *(NL)* to an EoE phenotype through the production of TSLP by the esophageal epithelium. TSLP-activated dendritic cells induce a robust T_H2 response and enhanced IL-13, which in turn mediates marked dysregulation of gene expression (the EoE transcriptome). Enhanced eotaxin-3 (CCL26) secretion by the esophageal epithelium promotes eosinophil migration from the blood into the tissue. Eosinophil- and mast cell–derived TGF-β along with IL-13 act on fibroblasts within the lamina propria to secrete periostin *(POSTN)* and stimulate the fibrotic response. Loss of *FLG* expression, partially because of IL-13 overproduction, genetic variants, or both, might further enhance or even predispose patients with EoE to antigen exposure and exacerbate T_H2 inflammation.

performed (in essence, boosting the statistical power). A hint at what SNPs or gene loci might hold potential significance in these future studies can be gained by means of investigation into those that did not reach the statistical threshold for significance from the previous GWAS.[52] For instance, SNPs in signal transducer and activator of transcription 6 *(STAT6)*, the major signaling molecule downstream of IL-13 signaling, and in the aforementioned *DSG1*, which is largely resistant to steroid-dependent regulation in patients with EoE, are highly associated with EoE but under the genome-wide significance threshold.[52]

CONCLUSIONS AND FUTURE DIRECTIONS

In just over 10 years since the recognition of EoE as a distinct inflammatory disorder, the rapid progress toward characterizing the disease on multiple fronts has underscored its complexity. We now have insight into the natural history of EoE, its strong association with specific ethnicities and sexes, the genetic and environmental factors involved, and the molecular pathogenesis of the disease (Fig 1). Moreover, the burst of data illustrating EoE risk variants in *CCL26*, *TGFB1*, *TSLP* and *CRLF2*, and *FLG* provide insight into the upstream mechanisms that regulate the expression of genes that are operational (and likely synergistic) in multiple aspects of EoE pathogenesis (Fig 2). For instance, perturbations in the TSLP signaling pathway as a result of variants either increasing *TSLP* gene expression or altering receptor function can amplify innate inflammatory responses to food antigens. Moreover, prolonged *CCL26* expression might further enhance eosinophil recruitment and TGF-β1 secretion to exacerbate tissue remodeling. Variants affecting *FLG* expression might disrupt normal esophageal barrier function and result in increased antigen exposure and affect overall tissue integrity. Despite these advances, much work remains in terms of identifying true casual variants and determining their mechanistic function in these pathways. A major initiative currently underway is to expand on the current genome-wide associated polymorphisms by increasing the number of genotyped patients with EoE; this will undoubtedly greatly expand the number of genetic loci linked with EoE risk. Moreover, deep sequencing efforts and extensive fine mapping of the established EoE susceptibility loci, such as *TSLP*, could identify rare variants, casual variants, or both that affect gene transcription.

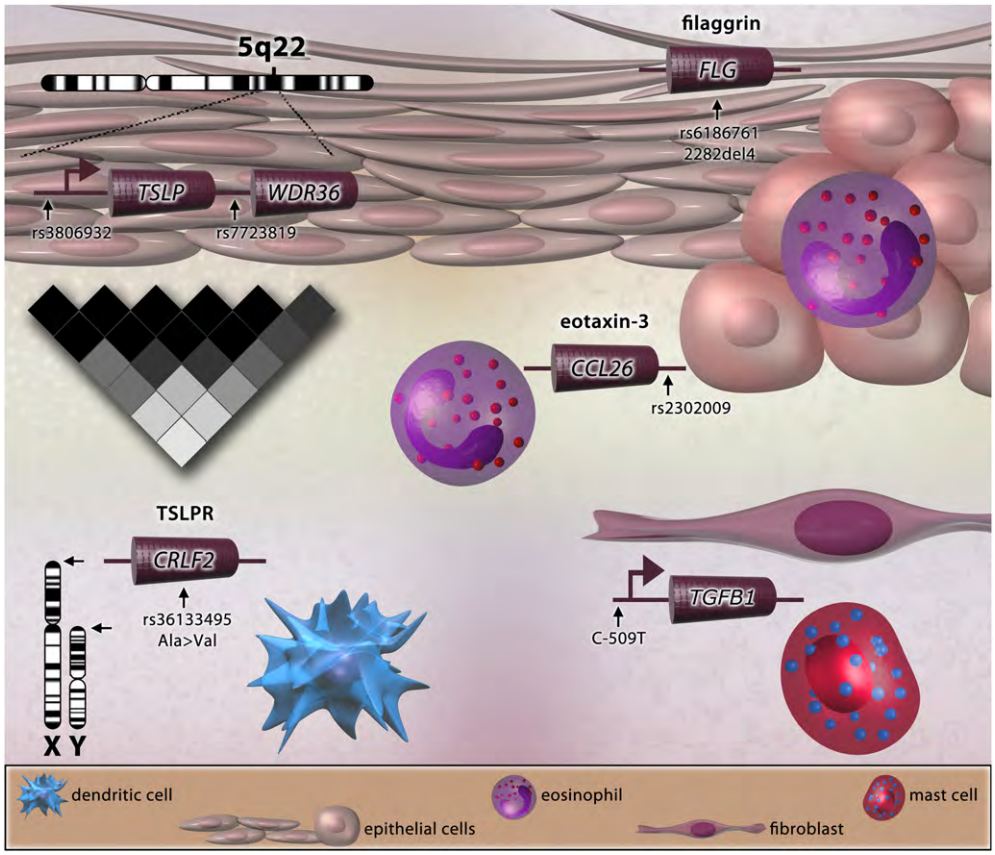

FIG 2. Genetic risk variants in patients with EoE. EoE risk variants near *TSLP* and in the TSLP receptor gene *(CRLF2)* highlight a potential role for the TSLP pathway in EoE. SNPs in other key genes, such as *CCL26*, *TGFB1*, and *FLG*, can affect multiple aspects of EoE pathogenesis, including eosinophil chemotaxis, fibrosis and smooth muscle dysfunction, and decreased esophageal barrier function, respectively.

An additional area to be explored in EoE heritability will be the role of ***epigenetics***, which can be defined as the study of heritable changes in gene expression that are not associated with DNA sequence variations, which can include noncoding RNAs, histone modifications (acetylation and methylation), and DNA methylation.[77] Importantly, because these genomic alterations can be influenced by external stimuli, such as diet and drugs, epigenetics can provide insight into the complex interactions between environmental exposures and disease-associated genes. The profiling of global epigenetic changes in large disease cohorts has already yielded promising results for cancer, cardiovascular disease, obesity,[77] and asthma.[78] Because EoE is also influenced by environmental antigen exposure, the uncovering of an EoE epigenome through microRNA arrays, DNA methylation profiling, and ***chromatin immunoprecipitation*** sequencing technologies will provide a critical link to the global gene transcriptional changes already known to occur in patients with EoE. Recent data have already indicated that IL-13 can elicit acetylation changes to histone H3 at the *CCL26* promoter in esophageal epithelial cells, implicating that epigenetic modifications represent a novel mechanism of gene regulation in patients with EoE.[79]

The pivotal role of the esophageal epithelium in patients with EoE and the transcriptional changes that occur within different stratified layers of the epithelium provide potential opportunities for noninvasive biomarkers for EoE. Laser-capture microscopy allows for the isolation of specific cell types from minute sections of tissues that can subsequently be subjected to microarray or mass spectrometric analysis. Such techniques have already been used to define the transcriptomes[80] and ***proteomes***[81] of the various esophageal layers in patients with Barrett esophagus. Identification of an EoE-specific transcript profile specific to the suprabasal epithelium might yield diagnostic targets from the skin or oral mucosal samples.

In conclusion, it is remarkable how the genetic dissection of EoE susceptibility has uncovered key pathways that are now being considered for treatment strategies. For example, our findings identify new targets for antibody neutralization strategies (eg, anti–IL-13) and specific cell types for directed therapy, such as mast cells and epithelial cells, which also supports the clinical value of topical steroid therapy. Therefore, over the next 10 years, further unraveling of the genetic and environmental factors that compound EoE holds great promise for the future development of novel and highly effective therapies.

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Key concepts

- **EoE is an emerging inflammatory disease that is clinically and causatively distinct from GERD.**
- **Genetic predisposition and food antigen exposure contribute to EoE.**
- **EoE exhibits a familial inheritance pattern and is more common among white subjects and male subjects.**
- **Microarray analysis of the patients' esophageal biopsy specimens has defined an EoE transcriptome that is also highly inducible by IL-13 *ex vivo*.**
- ***CCL26* is the most highly induced gene in the EoE transcriptome.**
- **Candidate gene studies have identified genetic variants in *CCL26*, *TGFB1*, *TSLP*, and *FLG* associated with EoE risk.**
- **A coding SNP in the TSLP receptor gene *CRLF2*, which is encoded on the X and Y chromosomes, is significantly associated with disease risk in male patients with EoE.**
- **A GWAS on 351 patients with EoE identified the 5q21 locus encoding *TSLP* and *WDR36* as an EoE susceptibility locus.**

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627 Genetic Risk Variants for Celiac Disease in the IL2/IL21 Locus are Associated with Eosinophilic Esophagitis

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RATIONALE: Eosinophilic esophagitis (EE) is a recently recognized food antigen-driven, Th2-associated inflammatory disease of the esophagus with increasing prevalence worldwide. Celiac disease is an autoimmune disorder also characterized by food hypersensitivity with concomitant gastrointestinal inflammation. Interestingly, EE and celiac disease have been reported to co-occur in some patients; however, direct evidence for a shared genetic basis in both diseases is lacking. Recent genetic data have identified non-HLA susceptibility loci associated with celiac disease, including polymorphisms in the IL2/IL21 locus. Given the similar etiologies and familial inheritance patterns, we hypothesized that risk variants for celiac disease also associate with increased susceptibility for EE.

METHODS: We genotyped a cohort of 272 EE patients and 450 normal controls for polymorphisms previously linked with celiac disease. Immunofluorescent microscopy and morphometric analysis was used to measure IL21 receptor expression in patient esophageal biopsies.

RESULTS: A number of genetic variants on chromosome 4q26-27 in the IL2/IL21 region were found to have significant associations with EE. In particular, rs2893008 ($p = 0.015$, odds ratio = 0.52) and rs4295278 ($p = 0.028$, odds ratio = 1.99), which are located approximately 10kb and 6kb from the 5' region of IL21, respectively, were the two most highly associated variants in the locus. Immunofluorescent microscopy and morphometric analysis demonstrate a marked increase in IL21 receptor-expressing cells within the esophageal epithelium of EE patients compared to non-EE patients.

CONCLUSIONS: These data suggest common pathways are involved in celiac disease and EE and indentify a possible role for IL2 and/or IL21 signaling in EE.

628 Correlation Between Degree Of Eosinophilia On Esophageal Biopsy And Number Of Positive Skin Prick Tests To Foods In Patients With Eosinophilic Esophagitis

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RATIONALE: Eosinophilic esophagitis(EoE) is a heterogeneous disease that is frequently associated with atopy and multiple food sensitivities. Elimination diets have been shown to be useful in conjunction with or after medical therapy has failed. Allergen skin prick testing(SPT) can be helpful to guide dietary recommendations. There are currently no studies published regarding whether the degree of focal eosinophilia in the esophagus correlates with the number of positive food allergy tests in patients with EoE.

METHODS: We performed a retrospective chart review of patients referred to the outpatient Allergy and Immunology clinic at Children's Hospital of Pittsburgh of UPMC with a diagnosis of EoE. Data was collected from patients who had both esophageal biopsies and skin prick testing to foods.

RESULTS: Data were collected for 75 patients. Based upon the number of eosinophils/hpf, patients were divided into 3 separate groups: A<25(n=42), B=26-50(n=25), C >51(n=8). Mean age in each group was similar (A=9.95years, B=10.2years, C=11.75years). The percentage of patients with at least 1 positive SPT in each group were similar but trended downwards (A=45%, B=40%, C=25%). The mean number of positive SPT per patient in each group also trended downwards (A=2.3, B=1.4, C=0.38). A statistically significant difference was observed for the percentage of positive SPT per total SPT performed in each group (A=15%, B=8.4%, C=1.8%).

CONCLUSIONS: Our data suggests that a higher degree of focal eosinophilia on esophageal biopsy in patients with EoE are less likely to have positive SPT to foods.

629 Using Allergic Sensitivities to Define Three Phenotypes among Patients with Eosinophilic Esophagitis

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RATIONALE: Eosinophilic esophagitis (EE) is an increasingly recognized chronic disorder. The relevance of the allergic sensitivities to foods and inhalants seen in this disease, to the pathogenesis and natural history, remains unknown.

METHODS: In a cohort of 30 pediatric EE patients, we performed skin prick testing and measured serum levels of specific IgE to a panel of common foods and inhalants and to the carbohydrate determinants bromelain, galactose alpha-1,3-galactose, and N-glycolylneuraminic acid (Pharmacia CAP).

RESULTS: The overall geometric mean(GM) total IgE was 82.8 IU/ml. No specific IgE was detected in 30% of the patients (GM total IgE 24.3 IU/ml; median 40 eos/hpf on biopsy). Serum IgE to milk was detected in nine cases; though, only two of these had positive skin tests. A third group (30%) had multiple sensitivities to foods and pollens (GM total IgE 285 IU/ml). Tests for IgE to carbohydrate antigens were negative in all sera except two in the "multiple sensitivity" group. Esophageal eosinophils and peripheral blood eosinophils were not different among the three groups (Kruskall-Wallis, p=0.3 for both parameters).

CONCLUSIONS: We identified three phenotypes in our EE cohort- non-sensitized, milk sensitized, and those with multiple pollen allergies. The frequent occurrence of multiple associated sensitivities to grains, legumes, molds, and pollens suggests that cross reactive IgE antibodies to carbohydrates or proteins could be responsible; however, our testing for carbohydrate cross reactivity was negative. Because of the large number of patients with specific IgE to milk and negative skin tests, serum IgE measurements may be helpful in planning an avoidance diet.

630 Decreased Expression of Gut Homing Integrins on Peripheral Blood Th2 Cells from Eosinophilic Gastrointestinal Disease

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RATIONALE: Allergic eosinophilic gastroenteritis (AEG) is associated with food antigen specific Th2 cells. The purpose of this study was to evaluate whether food antigen specific Th2 cells in AEG preferentially express gut homing receptors that may contribute to eosinophilic tissue inflammation.

METHODS: Peripheral blood mononuclear cells from 5 subjects with AEG were studied. Gut homing integrin expression on peanut antigen specific T cells was measured using polychromatic flow cytometry with intracellular cytokine staining. Integrin expression was analyzed as the ratio between antigen specific cytokine producing Th2 cells and mainstream non-antigen specific cells. A ratio of one implies equivalent homing receptor expression.

RESULTS: alpha4beta7 was expressed at a ratio of 0.17 (range 0.03-0.42) in IL-5 expressing peanut antigen specific cells relative to mainstream cells. Parallel studies of beta7 expression confirmed these results (ratio 0.14, range 0.10-0.38). Similarly, in IL-13 producing cells, both alpha4-beta7 and beta7 were expressed at ratios substantially below one: 0.11 (range 0.03-0.18) and 0.08 (range 0.00-0.13) respectively. Gut homing integrin expression was also decreased in staphylococcal enterotoxin B activated cells (ratio 0.54), however it was significantly lower in peanut specific T cells (ratio 0.14, $p = 0.001$).

CONCLUSIONS: Contrary to our expectation, gut homing integrin expression by peanut antigen specific Th2 cells was consistently less than that of mainstream T cells. This suggests that food allergen specific T cells have been depleted from the circulation, possibly due to sequestering in inflammatory GI tissue.

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Supporting Data. N/A